Voice prosthetic valve failure due to biofilm formation

G.J. Elving

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Stellingen

behorende bij het proefschrift

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Groningen, 23 januari 2002

1. Wie biofilms op spraakprotheses onderzoekt met als doel bacteriën of gisten te identificeren die het klepmechanisme aantasten, moet zich rekenschap geven van het feit waar precies op de spraakprothese de te onderzoeken biofilm is verwijderd *(dit proefschrift).*

2. *Candida*soorten alleen zijn niet in staat tot ingroei in siliconenrubber spraakprotheses *(dit proefschrift).*

3. De levensduur van de eerste spraakprothese van laryngectomiepatiënten dient niet meegenomen te worden in levensduurstudies van spraakprotheses *(dit proefschrift).*

4. De "live/dead *Bac*Light stain" voor het determineren van dode en levende bacteriën in een biofilm op een biomateriaal met behulp van de Confocale Laser Scanning Microscoop functioneert niet goed.

5. De voortdurende strijd over wie zich eerste, zoveelste of laatste auteur mag noemen van een wetenschappelijk artikel staat echte multidisciplinaire samenwerking in de weg.

6. Kansen krijg je niet, die moet je creëren.

7. Het gebruik van antibacteriële schoonmaakmiddelen in de huishouding stelt de eigen afweer van mensen zwaar op de proef.

8. De MKZ-verklaring voor kleine herkauwers valt onder "ministeriële dwalingen".

9. Wat je wint in de liefde, verlies je in de kilometers (vrij naar veehouder Stouten).

10. In het kader van miltvuurbesmettingen krijgt het gezegde "Hij heeft zijn sporen verdiend" een wel erg wrange betekenis.

11. Vertragingen bij de spoorwegen leiden tot overvolle treinen, want treinen die eerder werden gemist worden nu vaak gehaald.

12. In de zegswijze "Hij heeft een tong als een scheermes" dient de tong niet als biologisch wapen te worden gezien.

13. Binnen de boomchirurgie is amputeren geen specialisme.

RIJKSUNIVERSITEIT GRONINGEN

Voice prosthetic valve failure due to biofilm formation

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Aan mijn ouders

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Chapter 1

Introduction and Aims

Elving GJ, Van der Mei HC, Van Weissenbruch R, Albers FWJ, Busscher HJ
Effect of antifungal agents on indwelling voice prosthetic biofilms
Current Opinion in Otolaryngology & Head and Neck Surgery 2000;8:165-8.
(reproduced with permission of Lippincott Williams & Wilkins)

Introduction

The inability to speak is the most disabling consequence of total laryngectomy. Different methods of rehabilitating the lost voice of laryngectomized patients have been developed. The insertion of a silicone rubber voice prosthesis in a surgically created tracheoesophageal shunt or fistula was a major step forward in the speech rehabilitation of laryngectomized patients and is now generally considered to be superior to any other form of substitute voice production, such as esophageal and electrolaryngeal speech.

Voice prostheses are not permanent implants, but need to be replaced when patients complain about leakage through or around the prosthesis, or increased efforts to produce tracheoesophageal speech. Continuous exposure to saliva, food, drinks, and the oropharyngeal microflora contributes to the rapid colonization by a mixed biofilm of bacteria and yeasts, leading to valve failure and frequent exchange of the implant. Explanted voice prostheses show not only biofilm formation on the implants,[1] as shown in Figure 1, but also ingrowth of yeasts, mainly *Candida* species, into the silicone rubber.[2]

Candida albicans is frequently isolated from the human oral cavity, yet few carriers develop clinical signs of candidiasis. Oral candidiasis reflects the ability of the yeast to colonize different oral surfaces and the variety of factors that predispose the host to *Candida* colonization and subsequent infection. The host's immune competence ultimately determines whether clearance, colonization or candidiasis occurs. In the case of laryngectomized patients, predisposing conditions for increased *Candida* colonization such as the underlying neoplastic disease, surgical and extensive drug therapy, reduced saliva flow rate as a side-effect of radiotherapy, prosthetic tooth replacement, and the presence of the prosthesis itself can be considered.

Use of antifungal agents

Oropharyngeal yeast decontamination by using amphotericin B lozenges and buccal bioadhesive slow-release tablets containing miconazole nitrate has been applied by various ear, nose and throat surgeons[3,4] to increase the lifetime of voice prostheses. These methods are especially applied in laryngectomized patients with prosthesis lifetimes of less than two months, although scientific evidence regarding the efficacy of antimycotics in these applications is lacking.

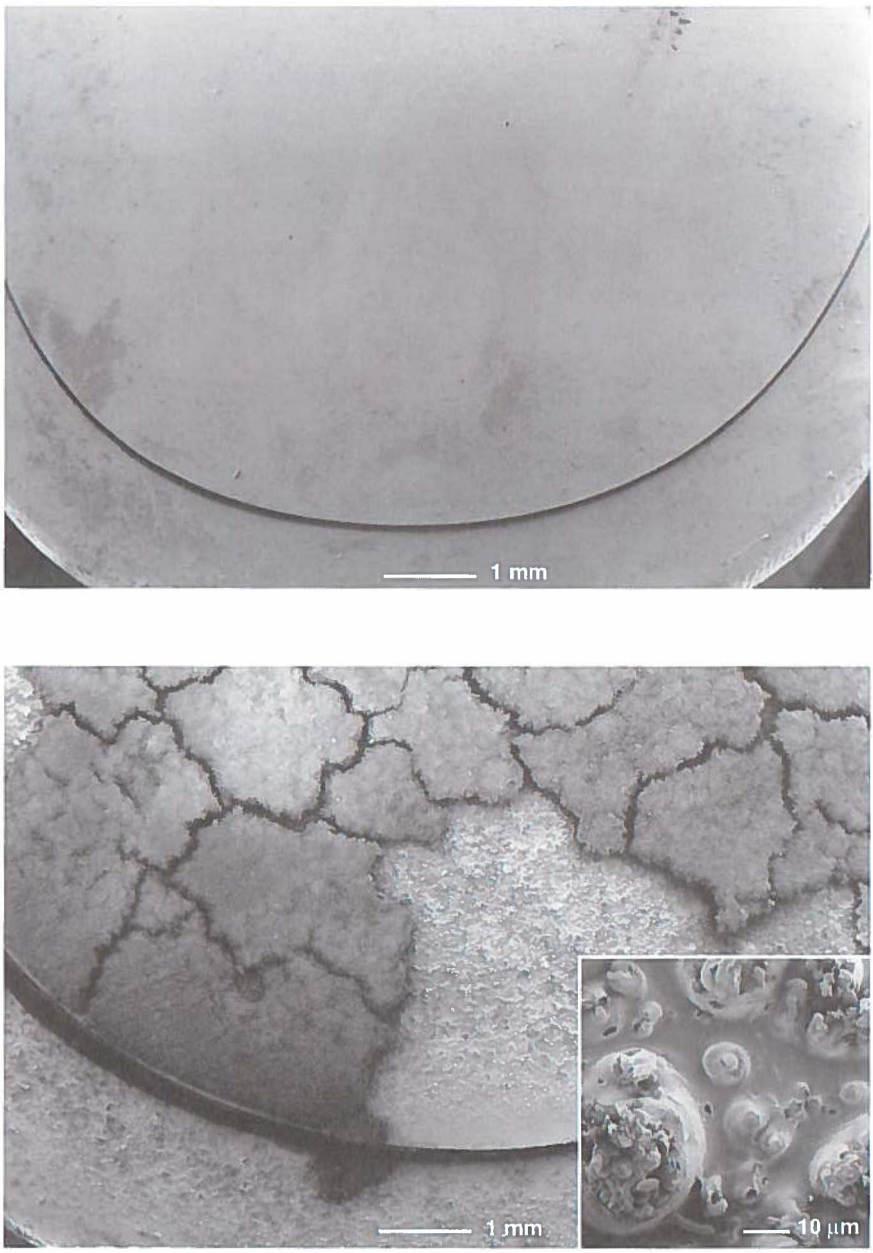

Figure 1. *Scanning electron micrographs of Groningen button silicone rubber voice prostheses, seen from esophageal side. Above, unused Groningen button voice prosthesis. Below, heavy biofilm formation on the valve side after use and ingrowth of biofilm organisms into the silicone rubber (insert).*

Recently, Ackerstaff *et al.*[5] performed a nonrandomized, multi-institutional, controlled clinical trial on a novel anterograde replacement method comparing the lifetime of the second-generation indwelling Provox 2 voice prosthesis (Atos Medical AB, Hörby, Sweden) with that of the original Provox voice prosthesis. In the four clinics participating in this study, patients requiring replacement within two months due to valve failures caused by *Candida* overgrowth were advised to use anti-*Candida* medication on a daily basis, either by swallowing the liquid medication or by applying it directly into the prosthesis with a cleaning brush. Of the 157 patients included in the prosthesis lifetime analysis, 45 patients (28.7%) reported that they regularly used an anti-*Candida* drug (nystatin, amphotericin B, or fluconazole). In a proportional hazard regression analysis, it was subsequently demonstrated that the use of anti-*Candida* medication was not significantly associated with the lifetime of the voice prostheses.

Conclusively, there is no compelling indication to prescribe antifungal agents for increasing the lifetime of voice prostheses in laryngectomized patients. Moreover, this prophylactic use of antifungal medication may contribute to the development of resistant yeast strains, whereas already the number of antifungal agents available is limited. However, these observations evoke the question "Why is not there an association between biofilm formation, prosthesis lifetime and the application of anti-*Candida* medication?"

Antimicrobial resistance of biofilms

Microbial biofilms are critical to the survival of the colonizing organisms in a wide variety of environments. The biofilm mode of growth protects microorganisms on voice prostheses against the host immune system and antibiotics or antimycotic agents.[6] These resistance properties of microbial biofilms have been attributed to an organization of the biofilm organisms within exopolymer matrices. Such exopolymers will chemically quench reactive biocides such as chlorine and peroxygens, and bind highly charged antibiotics, thereby protecting the organisms of the inner layer of the biofilm against antimicrobial agents. Therefore, microbial biofilms are hardly treatable because of the difficulties of antimicrobials in penetrating this biofilm. Moreover, in case of biofilms on silicone rubber voice prostheses the ingrowth of yeasts in the silicone rubber yields an extremely efficient shelter for the organisms against environmental attacks (see also Fig. 1). Recent work of Van Weissenbruch *et al.*[4] has shown that using antifungal agents in laryngectomized patients greatly decreases

prevalence of planktonic yeasts in saliva compared with those living in a biofilm in or on voice prostheses.

Alternatives for preventing biofilm formation on voice prostheses

Different strategies have been developed to prolong the lifetime of voice prostheses. Modification of the silicone rubber surface to discourage biofilm formation is an obvious strategy to prolong the lifetime of voice prostheses. Although voice prostheses will become covered by a conditioning film of adsorbed salivary components prior to the adhesion of bacteria or yeasts, experiments in the human oral cavity have demonstrated that the properties of this conditioning film are determined by the material itself.[7] By consequence, biofilm formation can be influenced by adjusting the properties of the voice prosthesis material or by surface modification.

Everaert *et al.*[8] demonstrated that biofilm formation on silicone rubber Groningen button voice prostheses over an evaluation period of approximately 2 to 8 weeks can be reduced by chemisorption of long perfluoro-alkylsiloxane polymer chains, owing to the high hydrophobicity and mobility of the chemisorbed polymer chains. However, the effect of this modification on the average lifetime of indwelling voice prostheses must still be determined. An invention has been patented[9] related to a way to inhibit microbial growth on the surface of a medical device such as a voice prosthesis by manufacturing the prosthesis of, or solely coating the surface with, a fluoropolymer. The patent claims an elongation of voice prosthesis lifetime by 133 days (or 28.1%) on average, but this claim is based on only three patients.

Within patient support groups in The Netherlands, laryngectomized patients have suggested that the consumption of buttermilk, containing antimycotic-releasing *Lactococcus lactis*, positively affects the lifetime of voice prostheses. This suggestion has been confirmed in an artificial throat model, in which the effects of daily buttermilk consumption on biofilm formation on silicone rubber voice prostheses have been simulated.[10,11] Similarly, Turkish yogurt containing *Streptococcus thermophilus* has been suggested to have such beneficial effects. Evaluations in the artificial throat model have furthermore indicated that the development of an oropharyngeal biofilm on silicone rubber voice prostheses can be delayed by exposure to caffeinated soft drinks[12] or suspensions of active probiotic bacteria, such as *L. lactis* 53 and *S. thermophilus* B.[13]

Many laryngectomized patients suffer from salivary dysfunction as a result of surgical therapy, radiation therapy, aging or medication. Low salivary secretion reduces the amounts of histatins in saliva, yielding better chances for opportunistic microorganisms such as *C. albicans*, because histatins are the most significant source of fungicidal activity in saliva.[14] Artificial salivary substitutes, commonly used by xerostomic patients and sometimes by laryngectomized patients, now mainly contain carboxymethylcellulose, animal mucins or xanthan, but these substances present an excellent vehicle for novel antifungal agents.[15] Promising antifungal agents are synthetic salivary peptides, which can possess bactericidal and fungicidal activities.[16,17] Moreover, these salivary peptides so far have not been associated with the development of microbial resistance.

Summary of conclusions

1. There is no compelling evidence that the prescription of antifungal agents will prolong the lifetime of voice prostheses in laryngectomized patients.
2. The prophylactic use of antifungal agents in laryngectomized patients contributes ultimately to the development of resistant strains. Thus alternative approaches are called for.
3. Alternative approaches to prolong the lifetime of silicone rubber voice prostheses may be found in silicone rubber surface modification, consumption of caffeinated soft drinks, diet supplementation with active, probiotic bacteria as can be found in certain dairy products, or salivary substitutes with synthetic antimicrobial peptides.

Aims of this thesis

The aims of this thesis are:

1. To make an inventory of different factors, including radiation therapy and microbial composition of voice prosthetic biofilms, on the lifetime of voice prostheses *in vivo* (Chapters 2 and 3).
2. To design an *in vitro* model to mimic biofilms as found on voice prostheses in laryngectomized patients with prosthetic lifetimes less than 3 to 4 months, and allowing to determine the influence of biofilms on the air flow resistance (Chapters 4 and 5).
3. To evaluate antimicrobials using this *in vitro* model that potentially delay the development of an oropharyngeal biofilm on silicone rubber voice prostheses (Chapters 6 and 7).

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Chapter 2

A Comparison of the Microbial Composition of Voice Prosthetic Biofilms in Patients Requiring Frequent Versus Infrequent Replacements

Elving GJ, Van der Mei HC, Busscher HJ, Van Weissenbruch R, Albers FWJ
A comparison of the microbial composition of voice prosthetic biofilms in patients requiring frequent versus infrequent replacements
Annals of Otology, Rhinology & Laryngology 2001, in press.

Introduction

Biomedical implants such as indwelling silicone rubber voice prostheses,[1] prosthetic heart valves,[2] indwelling urinary catheters,[2] silicone breast implants[3] and prosthetic joints[2] have contributed to the solution of several serious medical problems. Although the development of these biomaterials implants can be considered successful, a drawback of the introduction of biomaterials implants into the human body is that microorganisms have the tendency to adhere to biomaterials surfaces and cause biomaterials-related infections. Adhering microorganisms are organized within a biofilm, a unique environment in which they are protected against host defense mechanisms and influences of antimicrobial agents, as critical to their survival. Consequently, biomaterials-related infections inevitably lead to replacement or removal of an implant.

The formation of a biofilm on biomaterials surfaces occurs in consecutive steps. Directly after implantation, a biomaterials surface will become covered with a thin layer of organic origin, called "conditioning film".[4] In case of voice prostheses, the conditioning film consists of adsorbed salivary proteins.[5] Consequently, microorganisms will mostly adhere to this conditioning film and rarely to the surface of the biomaterial itself. At first, only single microorganisms adhere reversibly to the surface of an implant,[6] and subsequently, through the production of exopolymeric substances, become irreversibly bound. On silicone rubber voice prostheses, ingrowth of yeasts into the silicone rubber provides additional protection against environmental attacks,[7,8] next to the protection offered by the biofilm mode of growth.

Biofilm formation readily occurs on indwelling voice prostheses and a variety of different causative organisms have been identified, including *Candida* species, streptococci and enterococci. *Candida* species have been associated with biomaterials ingrowth and deterioration of voice prostheses.[8,9] In addition, Ell *et al.*[10] demonstrated an association between *Candida* in voice prosthetic biofilms and failure due to increased air flow resistance of valves after use for less than 75 days. Also, Ell *et al.*[11] found that valve leakage was associated with the prevalence of streptococci in biofilms in the valve and that enterococci in biofilms on the esophageal side were associated with high air flow resistance failure of voice prostheses within 75 days of use. Palmer *et al.*[12] suggested that the simultaneous presence of *Staphylococcus aureus* and *Candida* spp. in voice prosthetic biofilms stimulates valve failure.

Clinically, an impressive interindividual variation exists with regard to the lifetime of indwelling voice prostheses of laryngectomized patients. This extreme range in prosthesis

lifetimes among patients is reflected also in a recent study on the lifetimes of Provox voice prostheses, reported to be on average 115 days, with a range of 7 to 583 days.[13] Sometimes, for unknown reasons, patients can switch from the group needing frequent replacement to the group needing replacement once a year, or vice versa, without evident changes in medication or lifestyle. The causative factors for these extreme differences in prosthesis lifetimes are not entirely known, but it has been suggested that irradiation dose, volume of irradiated salivary gland tissue, residual salivary flow rate, time passed after irradiation or insertion of a prosthesis, surgical or drug therapy and prosthetic tooth replacement are involved.

All potential causes suggested above contribute to a disturbance of the oral microflora of the laryngectomized patient which may stimulate oral pathogens, such as yeasts to become dominant, as known, for instance in denture stomatitis.[14] Brown *et al.*[15] studied the influence of xerostomia after radiotherapy on human oral microflora and found, together with the saliva shutdown, a shift towards cariogenic microorganisms in the oral cavity after radiation, in particular increases in *Streptococcus mutans*, *Candida albicans* and species of *Staphylococcus* and *Lactobacillus*.

Although several studies have identified the strains and species involved in voice prosthetic biofilms, microbial analyses have not yet been aimed toward establishing a difference in biofilm composition of patients requiring frequent versus infrequent prosthesis replacements.

The aim of this study was to evaluate whether differences in microbial composition in the biofilm exist on indwelling silicone rubber voice prostheses, that have failed within 4 or after 9 months from the time of insertion.

Study design

Patient population and retrieval protocol

Over a 2 years evaluation period, 128 different patients visited the outpatient clinic of the Department of Otorhinolaryngology of the University Hospital Groningen, involving a total of 692 failed voice prostheses, which were all retrieved after replacement. Subsequently, only Groningen button voice prostheses were considered for this study that were removed because of increased airflow resistance hampering tracheoesophageal speech or leakage of food or liquids through the prosthesis. These voice prostheses were subdivided into two arbitrarily defined lifetime groups, representing extremes. Accordingly, 20 prostheses were entered in

the short lifetime group (18 men, 2 women, mean age 63.5 ± 10.6 years), corresponding with an implantation-period less than 4 months, while 18 prostheses were included in the extended lifetime group (15 men, 3 women, mean age 67.6 ± 8.2 years), comprising an implantation-period over 9 months.

Explanted prostheses were directly transferred to 60 ml plastic containers filled with reduced transport fluid (NaCl 0.9 g/l, $(NH_4)_2SO_4$ 0.9 g/l, KH_2PO_4 0.45 g/l, $MgSO_4$ 0.19 g/l, K_2HPO_4 0.45 g/l, Na_2EDTA 0.37 g/l, L-Cysteïne HCl 0.2 g/l, pH 6.8) for microbial analyses.

Isolation and identification of microorganisms

First, all explanted prostheses were macroscopically examined. Subsequently, the biofilm was removed from the valve sides of the prostheses by scraping and, after sonication for 60 s, the isolated microorganisms were serially diluted in reduced transport fluid. Subsequently, the microorganisms were plated on brain heart infusion (Oxoid, Basingstoke, Great Britain) agar plates for yeasts and blood agar plates for bacteria. After 3 days at 37°C in an aerobic incubator different yeast and bacterial colonies were distinguished based on visual inspection and subcultured.

Once pure fungal and bacterial cultures were obtained on a plate, the yeast strains were identified by the ID 32 C identification system (BioMérieux), while bacteria were first examined by light microscopy and Gram-staining. Subsequently, the bacterial strains were identified by the GP MicroPlate from Biolog for Gram-positive bacteria (Biolog, Hayward, CA, USA) and the GN MicroPlate from Biolog for Gram-negative bacteria.

Results

The explanted voice prostheses showed an enormous variety in macroscopically visible deterioration of the silicone rubber on the valve side. Within both the short and the extended lifetime group large brown deposits were seen on the esophageal side of the prostheses, but prostheses with a macroscopically clean surface were also retrieved.

The isolation of the microorganisms colonizing the 20 Groningen button voice prostheses of the short lifetime group resulted in a collection of 49 bacterial and 27 yeast isolates. Gram staining showed that 36 bacterial isolates were Gram-positive. The biofilms on the 18 Groningen button voice prostheses in the extended lifetime group comprised 43 bacterial isolates, of which 30 turned out to be Gram-positive and 25 yeast isolates.

Table 1 shows the frequency of isolation of the identified strains, comparing the short and the extended lifetime group. The 52 yeast strains were mainly *Candida* species (47 isolates), while other strains identified in the extended lifetime group were *Saccharomyces cerevisiae* (3 isolates) and *Trichosporon cutaneum* (2 isolates). Among the *Candida* strains, the most conspicuous difference between the short and extended lifetime group concerned *Candida albicans* I, *Candida tropicalis* and *Candida krusei*, which were all found more frequently in the short lifetime group than in the extended lifetime group. *Candida humicola*, *Candida glabrata*, *Candida parapsilosis*, *S. cerevisiae* and *T. cutaneum* were more frequently isolated in the extended lifetime group.

Most of the bacterial strains could be identified as *Rothia dentocariosa* (11 isolates), streptococci (8 isolates) and staphylococci (7 isolates). Particularly *R. dentocariosa* and staphylococci were isolated more frequently in the short lifetime group than in the extended lifetime group. The number of streptococci isolated in both lifetime groups did not differ in a meaningful way. Apart from the above strains, several other bacterial strains were isolated occasionally, that belonged to different genera (Table 1). The identification system employed could not classify 51 of the bacterial isolates, which were generally rod-shaped organisms (76%).

Discussion

In the present study, a difference in microbial composition of biofilms on indwelling silicone rubber voice prostheses failing within 4 months or after 9 months from the time of insertion was demonstrated.

Most of the microorganisms identified were yeast and bacterial strains normally present on the skin and in the oral cavity. Furthermore, a few bacterial strains originating from food and dairy products were identified. As in previous studies,[12,16,17] the most common microorganism colonizing the valve side of voice prostheses was *C. albicans*. In the short lifetime group, 70% of the voice prostheses retrieved were colonized with *C. albicans* I, whereas in the extended lifetime group the frequency of *C. albicans* I isolation was only 44%. *C. tropicalis* was only identified in the short lifetime group (20%) and never in the extended lifetime group. Van der Mei *et al.*[9] demonstrated *in vitro* that *C. albicans* and *C. tropicalis* both can be stimulated to grow into silicone rubber. Already within 14 days the initial biodeterioration of the silicone rubber by these yeast strains could be shown by scanning

Table 1. *Yeasts and bacteria isolated from 38 explanted Groningen button silicone rubber voice prostheses, subdivided into two groups according to their lifetime.*

	Isolation frequency (%)	
Yeast species	***Short lifetime***	***Extended lifetime***
Candida albicans 1	70	44
Candida humicola	10	17
Candida glabrata	10	17
Candida tropicalis	20	0
Candida parapsilosis	0	17
Saccharomyces cerevisiae	0	17
Candida krusei	10	6
Trichosporon cutaneum	0	11
Bacteria/genera species		
Rothia dentocariosa	45	11
Streptococcus	25	17
Streptococcus vestibularis	5	6
Streptococcus cricetus	0	6
Streptococcus suis	0	6
Streptococcus salivarius	5	0
Streptococcus mitis	5	0
Streptococcus pyogenes	5	0
Staphylococcus	25	11
Staphylococcus epidermidis	10	0
Staphylococcus aureus	5	0
Staphylococcus lugdunensis	5	0
Staphylococcus warneri	5	0
Staphylococcus cohnii	0	6
Staphylococcus intermedius	0	6
Escherichia coli	5	6
Stomatococcus mucilaginosus	0	6
CDC Group EF4	10	6
Photobacterium logei	10	0
Pediococcus pentosaceus	0	6
Kocuria kristinae	5	0
Leuconostoc mesenteroides	5	11
Bacillus laevolacticus	0	6

In the short lifetime group (lifetime < 4 months) 20 voice prostheses were included while the extended lifetime group (lifetime > 9 months) comprised 18 voice prostheses.
The isolation frequency is defined as the number of Groningen buttons on which a given strain was found relative to the total number of Groningen buttons in the group.

electron microscopy. In a longitudinal study by Neu *et al.*[18] involving ESKA-Herrmann prostheses, it was found that *C. albicans* is predominantly present in biofilms during the first 8 days after insertion, while *C. tropicalis* was only isolated from biofilms on voice prostheses more than 8 days after insertion. A major difference between the present study and the one by Neu *et al.*,[18] however, is constituted by the fact that here we only considered prostheses removed for clinical reasons, while Neu *et al.*[18] replaced prostheses at fixed points in time to demonstrate a possible colonization sequence in the absence of clinical reasons for replacement.

In this study, *R. dentocariosa* was the most frequently isolated bacterial strain in the short lifetime group, suggesting its association with prosthesis failure. Also staphylococci were most frequently isolated in the short lifetime group. Interestingly, Palmer *et al.*[12] found that 50% of Blom-Singer prostheses failing within 60 days, were colonized by *S. aureus*, while in the present study *S. aureus* was identified only once in the short lifetime group. Palmer *et al.*,[12] however, removed Blom-Singer valves, which can be cleaned daily by the patient while cleaning of Groningen button voice prostheses by patients is not usual. Furthermore, Palmer *et al.*[12] studied the biofilm of the complete Blom-Singer prosthetic valve, including the shaft and the tracheal side, which might well be colonized by different strains and species than the valve side of a voice prosthesis as examined in the present study.

It has been suggested that adhesion of bacteria to voice prostheses is a prerequisite for the subsequent colonization by yeasts.[18] Such a sequence in colonization by bacteria and yeasts has been described too for denture stomatitis.[14] Recently, Millsap *et al.*[19] studied *in vitro* adhesion of yeasts suspended in saliva to silicone rubber with and without adhering bacteria present on the silicone and demonstrated that most bacterial strains suppressed adhesion of yeasts. However, *C. albicans* and *C. tropicalis* adhered in higher numbers to silicone rubber with adhering *R. dentocariosa* or *S. aureus* present than in their absence. It could be concluded, that adhesive interactions between these two bacterial and yeast strains play a crucial role in the development of a biofilm. This conclusion is supported here by the observation that *R. dentocariosa* is the predominant strain colonizing prostheses in the short lifetime group. The role suggested by Millsap *et al.*[19] for *S. aureus* in stimulating adhesion of yeasts is in line with the association between *S. aureus* prevalence and prosthesis failure reported by Palmer *et al.*[12] As *R. dentocariosa* and *S. aureus* seem to emerge as bacterial strains stimulating colonization of voice prostheses by yeasts leading to their failure, exclusion of these two bacterial strains from the oral microflora, by selected antibiotics or

salivary peptides might well be more effective than the currently applied antimycotic regime, with no proven clinical efficacy.[20]

From the results of this study it can be concluded that *C. albicans* I, *C. tropicalis* and *R. dentocariosa* play a significant role in the early failure of Groningen button silicone rubber voice prostheses. Furthermore, it is of interest to conclude that the retrieval protocol, type of voice prosthesis and microbiological sampling areas on retrieved voice prostheses impact the microbial composition of the biofilms formed and the strains and species identified in them.

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Chapter 3

The Influence of Radiotherapy on the Lifetime of Silicone Rubber Voice Prostheses in Laryngectomized Patients

Elving GJ, Van Weissenbruch R, Burlage FR, Busscher HJ, Van der Mei HC, Albers FWJ
The influence of radiotherapy on the lifetime of silicone rubber voice prostheses in laryngectomized patients
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Introduction

In 1997, a total of approximately 700 laryngeal cancers were diagnosed in The Netherlands, representing 1% of all new cancers. The incidence of laryngeal cancer in women versus men is 1:6. In the USA approximately 11,500 patients are annually diagnosed with laryngeal cancer, with a ratio of women versus men of 1:4. Depending on tumor stage, treatment of laryngeal cancer consists of radiotherapy, surgery or both. Radiotherapy as a primary treatment is applied to approximately 70% of the patients, while the remaining 30% are initially treated by total laryngectomy. One of the disabling consequences of total laryngectomy is the inability to speak, but different methods of postlaryngectomy voice restoration are available. The use of a silicone rubber voice prosthesis inserted in a tracheoesophageal fistula or shunt is known as the most successful method.[1-3]

Although radiotherapy has a high rate of success in laryngeal cancer, a drawback of radiotherapy is that radiation also causes irreversible side effects in healthy tissues. Frequently, parts of the oral cavity and the major salivary glands are incorporated in the radiation field, which may lead to mucositis or hyposalivation.[4] Oral complications during or after radiotherapy are directly correlated with the irradiated volume of salivary gland tissue.[5] In contrast to mucositis which is a reversible side effect, hyposalivation is an irreversible effect especially in patients with head and neck cancer when the major salivary glands are included in the radiation field and receive a cumulative radiation dose of more than 40 Gray.[6] The flushing effect of salivary flow is the most important protector of the oral tissues against pathogenic microorganisms.[7] However, besides hyposalivation, irradiation of salivary glands also results in a change of salivary properties, such as a reduced pH, buffer capacity and the presence of specific non-immune and immune defense factors. The non-immune factors comprising lysozyme, lactoferrin, histatins and cystatins possess antibacterial, antifungal and antiviral properties.[8] Consequently, oral candidiasis often occurs during or shortly after radiotherapy as a result of hyposalivation and an altered salivary composition.[9-11]

A serious problem in laryngectomized patients with a voice prosthesis inserted, is the limited lifetime of the prosthesis of about 3 to 4 months.[12] Voice prostheses become covered with a biofilm, composed of bacteria, yeasts and their excreted organic matter, causing leakage of liquids or an increased air flow resistance resulting in replacement of the prosthesis.[13,14] Although replacement is a simple procedure carried out in the outpatient department, most patients experience replacements as unpleasant. Moreover, recurrent

replacements can cause damage to the tracheoesophageal fistula possibly leading to scar tissue formation, stenosis or insufficiency. Clinically, impressive interindividual differences in lifetime exist with regard to the lifetime of indwelling voice prostheses.

The aim of this study is to determine a possible relationship between voice prosthetic lifetime in laryngectomized patients and the irradiation dose applied to the neck node levels (field of the neck), in which the major salivary glands are partially included. Furthermore, the relationship between voice prosthetic lifetime and the irradiation dose applied to the primary tumor site was studied. To this end, a retrospective analysis is performed on 101 patients after total laryngectomy.

Patients and methods

Data collection

The records of 101 patients who underwent a total laryngectomy between January 1993 and November 1999 at the Department of Otorhinolaryngology of the University Hospital Groningen, The Netherlands were analyzed. The following parameters from the records were obtained: age; sex; radiotherapy; radiation fields (neck, tumor) and irradiation dose per field; tumor site; TNM classification and valve insertion. For each valve insertion, the interval of subsequent valve replacements and type of prosthesis were recorded.

Patients and treatment

In the Department of Otorhinolaryngology of the University Hospital Groningen, treatment of laryngeal cancer also depends on staging based upon the TNM classification. In small T1 and T2 tumors, the treatment of first choice is radiotherapy. Total laryngectomy, usually followed by (postoperative) radiotherapy, is generally performed in more extensive T3 and T4 tumors. In cases of recurrence after primary treatment with radiotherapy, patients will undergo salvage surgery.

Patients were treated by conventional fractionated radiotherapy in 2 Gray fractions, 5 days per week. The volume of irradiated tissue can generally be divided in two fields, the neck and the tumor site. Whenever the neck is irradiated, mostly reduction of the field occurs after 46 Gray to give a boost at the primary tumor site. The total dose to the primary tumor site ranges from 60 to 70 Gray in 2 Gray fractions, 5 days per week. The field of the neck includes the whole submandibular glands and, depending on tumor site and stage, a part of the

parotid glands (no more than 50% of their volume) will be included. In T1 tumors, the major salivary glands are generally not included in the radiation field and in T2 tumors it is dependent on tumor site and accurate classification of the tumor. In T3 and T4 tumors, on the other hand, the whole submandibular glands and up to 50% of the parotid glands are usually part of the radiation field. Lymph node metastases are treated by an additional unilateral or bilateral neck dissection.

Voice prosthesis and placement

Tracheoesophageal puncture and insertion of the primary voice prosthesis together with myotomy of the cricopharyngeal and pharyngeal constrictor inferior muscles is performed in all patients at the time of surgery. At present, the first inserted voice prosthesis is always a "Low Resistance" Groningen button voice prosthesis. At the end of the lifetime of the first inserted voice prosthesis, replacements are usually performed at the outpatient department with a self-retaining type prosthesis by the patients preference. In the period before 1996, all patients were routinely fitted with a "Low Resistance" Groningen button voice prosthesis, but nowadays the application of Provox 2 voice prostheses are quite common.

Statistics

Statistical processing was performed with the computer-assisted program SPSS 10.0 for Windows using the Mann-Whitney Test, and accepting $p < 0.05$ as statistically significant.

Results

Patients

After irradiation and laryngectomy, the group of patients consisted of 87 men and 14 women. The mean age at laryngectomy was 63 years (range: 39 to 86 years). The mean age of male patients was 63 years and of female patients 60 years. The follow-up period since the first insertion of a voice prosthesis varied from 1 to 106 months (mean: 26 months). The most common tumor site was the glottis (n=44, 43.6%) followed by supraglottis (n=39, 38.6%), hypopharynx (n=13, 12.9%), transglottis (n=4, 4%) and oropharynx (n=1, 1%). The tumors were classified as T1 in 23.8% of the cases and T2, T3 and T4 in 26.7%, 13.9% and 35.6% of the cases, respectively. 23 patients (22.9%) had nodal metastasis at the time of diagnosis, classified as N1 in 11.9% of the cases and as N2 in 10.9% of the cases.

During the course of this study, 24 patients died and 16 patients abandoned voice prosthetic speech restoration for several reasons, followed by spontaneous or surgical closure of the tracheoesophageal fistula. In these cases, all accomplished voice prosthetic replacements were included in this study.

Lifetime

In this study, 685 voice prosthesis replacements were included: 377 "Low Resistance" Groningen button voice prostheses, 296 Provox 2, and 12 original Provox voice prostheses. The original Provox prostheses were not taken into consideration due to the low number. The average lifetime of the first inserted "Low Resistance" Groningen button voice prosthesis, used immediately after laryngectomy, was 180 days (range: 15 to 1426 days). On average, 7 prostheses were replaced in each patient (range: 1 to 29 replacements).

The mean valve lifetime per patient was 132 days, with a minimum of 12 and a maximum of 551 days. Routine use of the "Low Resistance" Groningen prosthesis yielded a shorter average lifetime per patient of 137 days than of the first prosthesis after laryngectomy ($p < 0.05$). The average lifetime per patient of the Provox 2 voice prosthesis was 90 days, which presents no statistically significant difference with the "Low Resistance" Groningen button voice prosthesis, provided that the first Groningen button voice prosthesis, as used by all patients, is excluded from the analysis.

Radiotherapy

In 64 patients (63% of all cases), a laryngectomy was performed because of recurrence after radiotherapy (prelaryngectomy radiotherapy group). In 31 patients (31%), the total laryngectomy was followed by radiotherapy (postlaryngectomy radiotherapy group). Only 6% of the patients did not receive any radiotherapy.

From the total of 101 laryngectomized patients, 71 patients received radiotherapy to the field of the neck (including the primary tumor site), in which the whole submandibular glands and up to 50% of the parotid glands are included, with a mean cumulative dose of 47 Gray (range: 30 to 64 Gray), in 2 Gray per fraction. The radiotherapy of the remaining patients (n=30) was restricted to the primary tumor site (n=24). In the residual group (n=6) no radiotherapy was necessary.

The mean cumulative irradiation dose applied to the primary tumor site was 64 Gray (range: 30 to 70 Gray), in 2 Gray per fraction (n=95 patients). The remaining patients (n=6) did not have any radiotherapy.

Table 1. *Distribution of the irradiated laryngectomized patients per tumor classification comparing the group of patients who received a tumor dose of ≤ 50 Gray and the group who received a tumor dose of ≥ 60 Gray. Also the laryngectomized patients per tumor classification, who received either or not irradiation of the neck are mentioned.*

	Irradiation of the neck		***Tumor dose***	
T classification	***Yes***	***No***	***≤ 50 Gray***	***≥ 60 Gray***
T1	5	19	0	24
T2	23	4	0	27
T3	12	2	2	10
T4	31	5	11	21

Relation between lifetime and radiotherapy

The mean lifetime of the first tracheoesophageal voice prosthesis in the prelaryngectomy radiotherapy group (n=64, 189 days) and the postlaryngectomy radiotherapy group (n=31, 150 days) were not significantly different. Furthermore, no significant difference was established comparing the mean voice prosthetic lifetime per patient, excluding the first prosthesis, for the prelaryngectomy radiotherapy group and the postlaryngectomy radiotherapy group. The not irradiated group of laryngectomized patients (n=6) was not taken into consideration because of the low number.

Also, the mean voice prosthetic lifetime per patient, excluding the first prosthesis, for the patient group who received irradiation of the neck and the group who did not receive irradiation of the neck, was not significantly different. Figure 1 shows the correlation between the mean voice prosthetic lifetime per patient, excluding the first prosthesis, and the irradiation dose applied to the primary tumor site (tumor dose). The mean voice prosthetic lifetime per patient, excluding the first prosthesis, for the patient group who received a tumor dose equal or less than 50 Gray (n=12) was significantly ($p < 0.05$) longer than for the patient group with a tumor dose equal or more than 60 Gray (n=78). There were no significant influences of sex, age, mean number of prostheses per patient, nodal metastasis or tumor site. However, comparing the patient group who received a tumor dose equal or less than 50 Gray (n=12) with the patient group with a tumor dose equal or more than 60 Gray (n=78) for the T-classification of the tumor (see Table 1), there was a significant difference ($p < 0.05$).

Discussion

In the present study, a possible relationship between lifetime of voice prostheses in laryngectomized patients and radiotherapy, subdivided in the cumulative radiation dose applied to the primary tumor site and, in case extensive neck node levels have been irradiated, the cumulative dose applied to the neck, was studied. Conspicuously, no relationship between voice prosthetic lifetime and irradiation to more extensive neck fields could be established. However, primary tumor doses exceeding 60 Gray significantly shortened the mean voice prosthetic lifetime per patient. Earlier, Trudeau *et al.*[15] identified possible complications associated with therapeutic radiation among laryngectomized patients with tracheoesophageal puncture for voice restoration, including leakage via tracheoesophageal fistula, stoma

stenosis, and the ultimate achievement of tracheoesophageal speech and found no differences between radiated and nonirradiated patients.

Irriadiation to extensive neck fields, including the submandibular glands, with a cumulative radiation dose of 47 Gray yields irreversible hyposalivation.[6] Although the submandibular glands are normally responsible for 70% of the total salivary flow in a non-stimulated situation, irradiation of submandibular glands did not influence the voice prosthetic lifetime after laryngectomy. Moreover, patients with T1 tumors, only irradiated to the primary tumor site, did not show more extended mean voice prosthetic lifetimes compared to the mean voice prosthetic lifetimes of patients with T2, T3 and T4 tumors, who received irradiation to more extensive neck fields, including the submandibular glands.

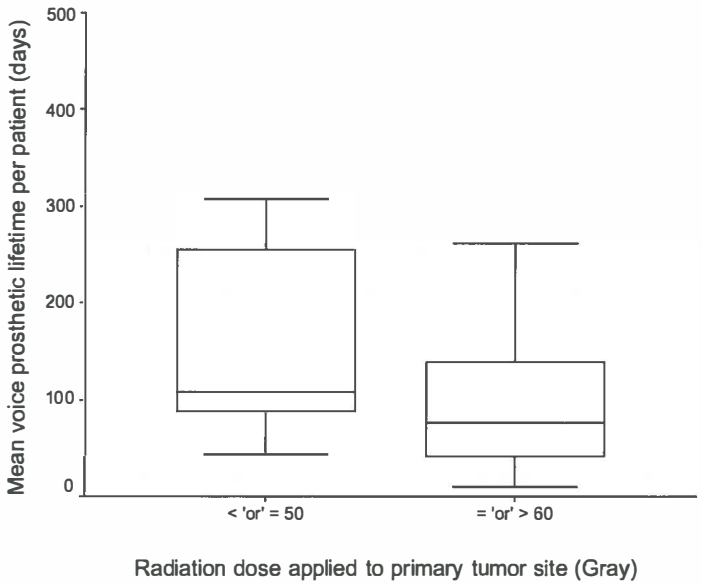

Figure 1. *The correlation between mean voice prosthetic lifetime per patient, excluding the first prosthesis, and the irradiation dose applied to the primary tumor site.*

High primary tumor doses were correlated with shortened prosthesis lifetimes, leading to the suggestion that limited lifetimes of voice prostheses are attributed to a local disturbance of the microflora of the pharyngoesophageal segment caused by a decrease in mucine production of the minor salivary glands of the pharyngoesophageal segment. Under healthy conditions, mucines function as a barrier against microorganisms and destruction of this barrier by radiotherapy could enable especially *Candida tropicalis*, *Candida albicans* and *Rothia dentocariosa*[16] to adhere to the pharyngeal epithelial surface and the valve surface of voice prostheses. Furthermore, within this mucine layer immunoglobulins are present protecting the pharynx against local infections.[7] Protection of the pharyngoesophageal segment during radiotherapy could be suggested in patients receiving a primary tumor dose equal or more than 60 Gray, but this therapy will not be sufficient considering possible infiltration of tumor into the parapharyngeal structures.

Conclusions

Summarizing, this study has identified an association between radiation on the primary tumor site with a dose equal or more than 60 Gray and limited lifetimes of voice prostheses. Based on these results and the fact that radiation of the pharyngoesophageal segment cannot be avoided, the application of a mucin-based artificial saliva could be an alternative method in order to increase the lifetime of voice prostheses by restoring the mucine barrier and therewith the quality of life of patients after laryngectomy.

Acknowledgment

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Chapter 4

Air Flow Resistances of Silicone Rubber Voice Prostheses after Formation of Bacterial and Fungal Biofilms

Elving GJ, Van der Mei HC, Busscher HJ, Van Weissenbruch R, Albers FWJ
Air flow resistances of silicone rubber voice prostheses after formation of bacterial and fungal biofilms
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Introduction

Silicone rubber voice prostheses are used to rehabilitate the voice of patients after surgical removal of a malignant tumor in the larynx area, a so-called laryngectomy. After laryngectomy (see Fig. 1), the respiratory tract is separated from the digestive tract and laryngectomized patients have to breath through an opening in the neck, a tracheostoma, while a one-way shunt-valve is placed between trachea and esophagus. The shunt-valve enables patients to speak by closing the tracheostoma with a finger and forcing air through the tracheoesophageal shunt into the esophagus, where remaining muscular structures act as pseudo vocal cords. The esophageal valve becomes colonized within 3 to 4 months on average by a biofilm, either causing leakage of liquids into the trachea or an increased air flow resistance and the prosthesis has to be replaced.[2-6]

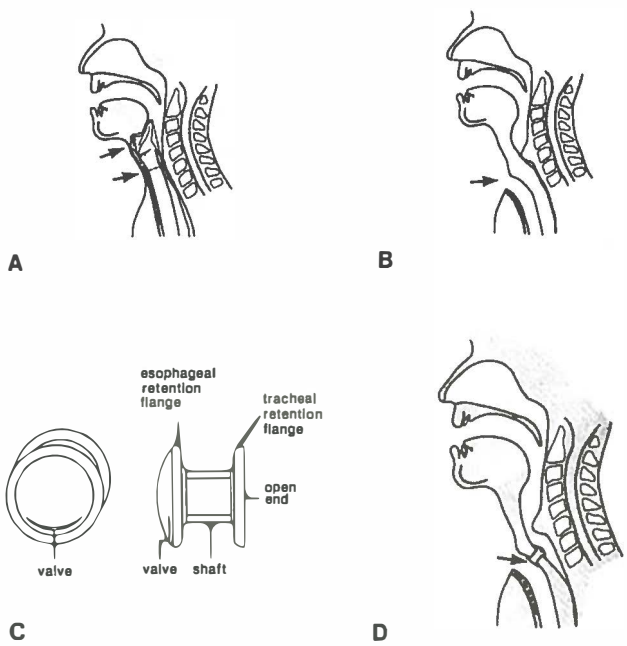


Figure 1. *A) Anatomy of the esophageal region before laryngectomy (arrows indicate parts to be removed). B) Anatomy after laryngectomy showing the separation of the airway and the digestive tract (arrow indicates tracheostoma). C) Sketch of the Groningen button silicone rubber voice prosthesis. D) Groningen button inserted into the tracheoesophageal shunt (arrow) (adapted from: Mahieu[1]).*

Since the first description of biofilms on voice prostheses by Mahieu *et al.*,[7] a large number of different microbial species have been isolated by various groups from voice prosthetic biofilms.[8-10] *Candida* species are generally held responsible for deterioration of the silicone rubber material of the prostheses as yeasts have been found growing into the silicone rubber.[7,11-14] Preventive measures have therefore been targeted mostly against the fungal components in these biofilms and patients have been put on an antimycotic regime for extended periods of time.[15-18] However, a drawback of long-term intermittent or continuous use of chemoprophylaxis is the induction of resistant strains. Moreover, there is no convincing evidence that prophylactic application of antifungal agents prolonges the lifetime of voice prostheses. Adjustment of dietary habits, including daily consumption of buttermilk, bioyogurts[19] or caffeinated soft drinks,[20] have all been suggested as measures to prolong the lifetime of voice prostheses through a reduction of biofilm formation.

However, it has also been suggested that an association exists between presence of particular bacterial strains in voice prosthetic biofilms and failure of voice prostheses. *Candida* and enterococci have been assumed to be jointly responsible for increasing the air flow resistance of voice prostheses after use for less than 75 days.[13,21] Streptococci have been suggested to cause valve leakage.[21] Although different strains and species work in concert in a voice prosthetic biofilm, these observations already indicate that not each organism in a voice prosthetic biofilm equally contributes to valve failure. Better identification of the species, causative for failure of a voice prosthesis, would allow better targeting of preventive measures against these organisms.

Biofilm formation on voice prostheses *in vitro* can be studied in the so-called artificial throat model,[22] which is based on the modified Robbins device. In the artificial throat (see Fig. 2), biofilms are grown on the esophageal side of a voice prosthesis during cycles of feast and famine at temperatures between 36°C and 37°C. Previously,[22] it has been shown that inoculation of the artificial throat with the total cultivable microflora from an explanted voice prosthesis yields biofilms that are electron-microscopically indistinguishable from biofilms formed in patients.

The aim of this study is to determine which bacterial or yeast strains contribute most to increases in air flow resistance of silicone rubber voice prostheses. To this end, biofilms consisting either of a bacterial or a yeast strain, isolated from explanted voice prostheses,[23] are grown on voice prostheses in the artificial throat model and the effects of these biofilms on air flow resistances are determined.

Materials and methods

Voice prostheses

"Low Resistance" silicone rubber Groningen button voice prostheses (see Fig. 1C) were kindly provided by Médin Instruments and Supplies (Groningen, The Netherlands) and were used throughout this study.

Biofilm formation

Groningen button voice prostheses were placed in five modified Robbins devices or artificial throats,[22] made of stainless steel (Fig. 2). Modified Robbins devices were autoclaved before use. Each artificial throat was equipped with two voice prostheses and maintained at temperatures between 36°C and 37°C, as in a laryngectomized patient.

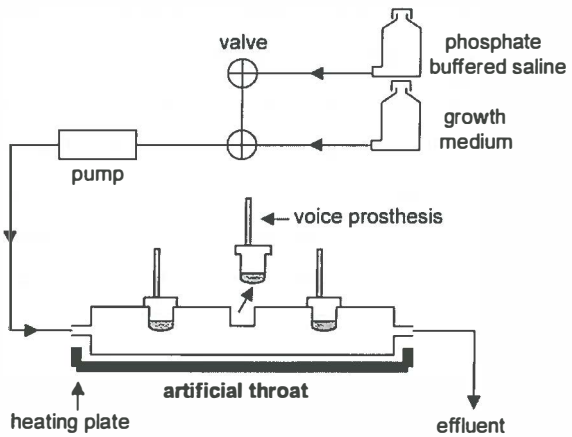


Figure 2. *Schematic presentation of the artificial throat, equipped with three Groningen button voice prostheses.*

Bacterial or yeast strains, previously isolated from explanted Groningen button voice prostheses from different patients and lifetimes varying from 1 to 29 months (see Table 1), were cultured in brain heart infusion broth (OXOID, Basingstoke, Great Britain) and used to inoculate the artificial throats during 5 h. Subsequently, a biofilm consisting either of a bacterial or yeast strain, was allowed to grow on the voice prostheses during 3 days by filling

the devices with growth medium. From day 4 till day 7 the artificial throats are flushed three times a day with 250 ml phosphate buffered saline (10 mmol/l potassium phosphate and 150 mmol/l NaCl, pH 7.0) and left drained, while at the end of each day the devices are filled with growth medium during half an hour and subsequently left overnight in the moist environment of the drained throats. Previously, this cycle of feast and famine has been demonstrated essential to grow biofilms with similar features as found on explanted prostheses. The tracheal sides of the prostheses were left in ambient air, similar to the situation with a stoma.

Measurement of air flow resistances

Compressed air was blown through each voice prosthesis prior to biofilm formation and as covered with a 7 days old biofilm of a given bacterial or yeast strain. Air pressures were varied between 10 and 20 cm H_2O and the resulting air flow (l/s) through the prosthesis was measured using a flow head; the flow head was calibrated with a Brooks flow meter. The pressure was measured with a pressure transducer, which was calibrated against a water manometer. The pressure range applied corresponds with clinically relevant conditions and yields a linear relationship between air pressure and flow. From the linear trajectory between air pressure and flow, an air flow resistance (cm H_2O.s/l) was calculated by linear regression analysis. As the air flow resistance of individual voice prostheses prior to biofilm formation differed, the effects of biofilms on the air flow resistance of a prosthesis, were expressed relative to the air flow resistance of a prosthesis prior to biofilm formation.

Evaluation of biofilms

On the eighth day of an experiment, voice prostheses were removed from the artificial throats after a final perfusion with 250 ml phosphate buffered saline. After measuring the air flow resistances of the prostheses as described above, biofilm formation on the valve side of one prosthesis was qualitatively assessed by scanning electron microscopy (SEM), while the second prosthesis was used to determine the number of colony forming units (CFU's) by plating of the biofilm on agar plates.

For electron microscopy, biofilm covered voice prostheses were flushed with 6.8% sucrose and 0.1 mol/l cacodylate buffer (pH 7.4), fixed and stained in 2% glutardialdehyde and 0.2% ruthenium red in 0.1 mol/l cacodylate buffer at 4°C and flushed again. Post-fixation and staining was carried out in 1% OsO_4 and 0.2% ruthenium red in cacodylate buffer by gently shaking for 3 h at room temperature. Buffer washes and dehydration involved the

following rinsing procedures: 20 min in 6.8% sucrose in 0.1 mol/l cacodylate buffer; 3x10 min bidistilled water; 20 min in respectively 30, 50 and 70% ethanol and 4x30 min in 100% ethanol. After critical-point drying with CO_2 for 4 h, the specimens were mounted on SEM stubs and sputter-coated with gold/paladium (15nm). SEM observations were taken, made using the JEOL 6301, with different magnifications at 15-25 kV.

In order to determine the number of CFU's in the biofilm, biofilms were removed by scraping and sonication and subsequently serially diluted. After plating bacteria on blood agar plates and yeasts on brain heart infusion agar, plates were stored at 37°C in an aerobic incubator for 3 days prior to enumeration.

Results

Air flow resistance

The air flow resistance of the Groningen button voice prostheses used prior to biofilm formation amounted 69 ± 16 cm H_2O.s/l, as averaged over all 96 prostheses involved in this study. The air flow resistance of prostheses increased slightly upon wetting, also in the absence of a biofilm and prostheses placed in the artificial throat after a similar perfusion scheme as during biofilm formation, showed an average increase in air flow resistance of 11 ± 5 cm H_2O.s/l.

Figure 3 shows pressure-flow diagrams of "Low Resistance" Groningen button voice prostheses prior to and after 7 days biofilm formation for *Streptococcus salivarius* GBJ 52/2A (Fig. 3A), *Rothia dentocariosa* GBJ 41/25B (Fig. 3B), and for the yeast strain *Candida tropicalis* GB 9/9 (Fig. 3C). The air flow resistance increases slightly after formation of a streptococcal biofilm, as indicated by the slope of the lines. However, 7 days old biofilms of *R. dentocariosa* GBJ 41/25B or *C. tropicalis* GB 9/9 yield much stronger increases in air flow resistance of a prosthesis.

Table 1 summarizes the increases in air flow resistance of "Low Resistance" Groningen button voice prostheses caused by 7 days old bacterial and fungal biofilms. All increases in air flow resistance due to biofilm formation that are significantly different (Student t test, $p < 0.05$) from the control (i.e. a wetted prosthesis), are indicated by an asterisk. Voice prosthetic biofilms formed on the esophageal side by biofilms of both *S. salivarius* strains showed a negligible increase in the voice prosthetic air flow resistance of 10

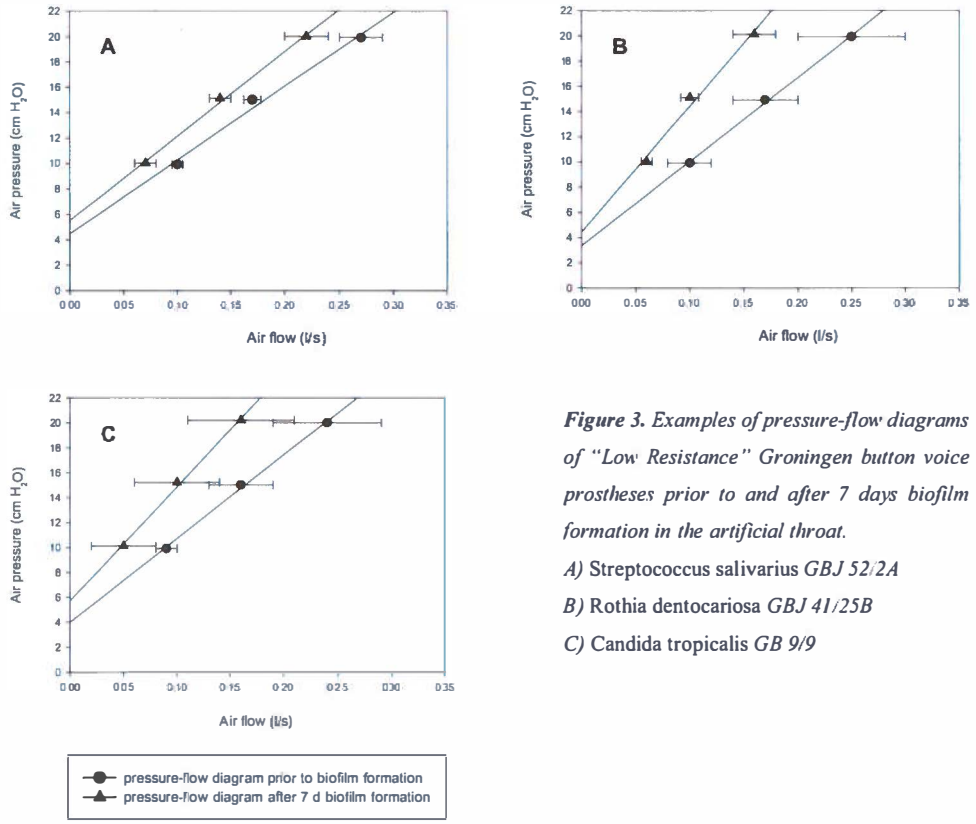


Figure 3. *Examples of pressure-flow diagrams of "Low Resistance" Groningen button voice prostheses prior to and after 7 days biofilm formation in the artificial throat.*

A) Streptococcus salivarius *GBJ 52/2A*

B) Rothia dentocariosa *GBJ 41/25B*

C) Candida tropicalis *GB 9/9*

to 12 cm H_2O.s/l, which is not statistically different from the increase observed upon wetting a prosthesis. The increases in voice prosthetic air flow resistance caused by biofilms of *Stomatococcus mucilaginosus* GB 16/3, *Streptococcus anginosus* GBJ 27/5C and the enterococcal and staphylococcal biofilms, except for *Staphylococcus aureus* GB 2/1, were about twice the increases caused by the biofilms of both *S. salivarius* strains and varied between 17 and 26 cm H_2O.s/l (*Staphylococcus intermedius* GBJ 42/29A). The yeast strains *C. tropicalis* and *Candida albicans* significantly increased the air flow resistance of the prostheses by 21 to 27 cm H_2O.s/l. Both *R. dentocariosa*, *Escherichia coli* GBJ 32/28D and *S. aureus* GB 2/1 biofilms caused the largest increases in air flow resistance (34 ± 15 cm H_2O.s/l for *S. aureus* GB 2/1).

Table 1. *The number of bacterial or yeast colony forming units per cm^2 on the esophageal surface of "Low Resistance" silicone rubber Groningen button voice prostheses after 7 days biofilm formation in the artificial throat, together with the increases in air flow resistance caused by these biofilms. All experiments were carried out in triplicate with separately cultured strains.*

Strains	***Colony forming units*** $(cm^{-2})^a$	***Increase of air flow resistance*** $(cm\ H_2O.s/l)^b$
Staphylococcus aureus GB 2/1	9×10^6	$34 \pm 15^*$
Rothia dentocariosa GBJ 41/25B	1.4×10^6	$33 \pm 8^*$
Escherichia coli GBJ 32/28D	1.4×10^7	$29 \pm 9^*$
Rothia dentocariosa GBJ 52/2B	4.5×10^6	$28 \pm 13^*$
Candida albicans I GBJ 33/9D	2.3×10^6	$27 \pm 11^*$
Staphylococcus intermedius GBJ 42/29A	3.2×10^6	$26 \pm 10^*$
Staphylococcus epidermidis GB 9/6	4.5×10^6	$25 \pm 14^*$
Candida albicans I GBJ 13/4A	4.5×10^6	$24 \pm 5^*$
Candida tropicalis GB 9/9	5×10^7	$21 \pm 6^*$
Enterococcus faecium 603	2.3×10^5	$19 \pm 3^*$
Streptococcus anginosus GBJ 27/5C	9×10^4	18 ± 14
Enterococcus faecalis 1131	2.7×10^6	$17 \pm 4^*$
Stomatococcus mucilaginosus GB 16/3	4.1×10^4	17 ± 8
Streptococcus salivarius GB 24/9	1.4×10^4	12 ± 6
Streptococcus salivarius GBJ 52/2A	1.4×10^7	10 ± 2
None	0	11 ± 5

[a] *Standard deviation over three separate experiments amounts 20-30%.*

[b] *Asterisks indicate significant differences (Student t test, $p < 0.05$) from the control (i.e. a wetted prosthesis), while ± indicate standard deviations.*

CFU's and scanning electron microscopy

Table 1 also summarizes the number of CFU's found in the biofilms for the different strains and species employed. Interestingly, no relation exists between the amount of organisms present on a prosthesis and the increases in air flow resistance measured. Figure 4 shows electron micrographs of voice prostheses removed from the artificial throat after 7 days with biofilms of different strains. The biofilm of *S. salivarius* GB 24/9 (Fig. 4A) is characterized by large clusters of cocci scattered over the valve side (concurrent with a negligible increase in air flow resistance, see Table 1), while the biofilm formed by *S. epidermidis* GB 9/6 (Fig. 4B) has a more slimy appearance and individual organisms seem connected by slime threads. Also the *E. coli* GBJ 32/28D biofilm (Fig. 4C), causing a strong increase in air flow resistance (see Table 1), consists of massive patches of biofilm and organisms appear linked by slime threads. Figure 4D shows yeast buds and mycelia typical for *Candida* organisms. Note that *C. albicans* I GBJ 13/4A does not form a contiguous biofilm, as do *S. epidermidis* GB 9/6 and *E. coli* GBJ 32/28D.

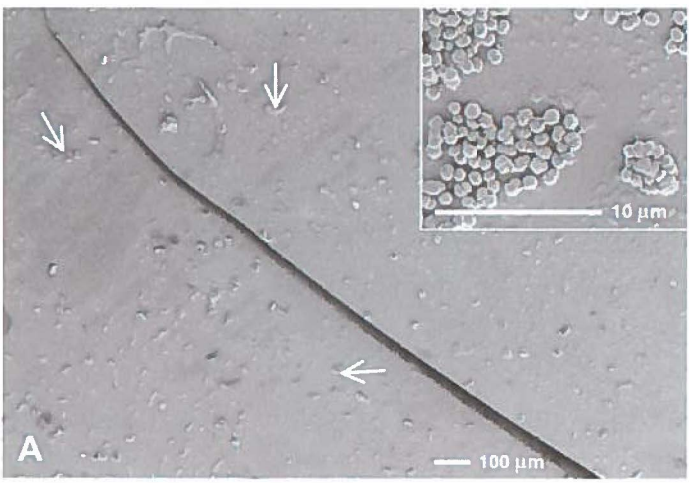
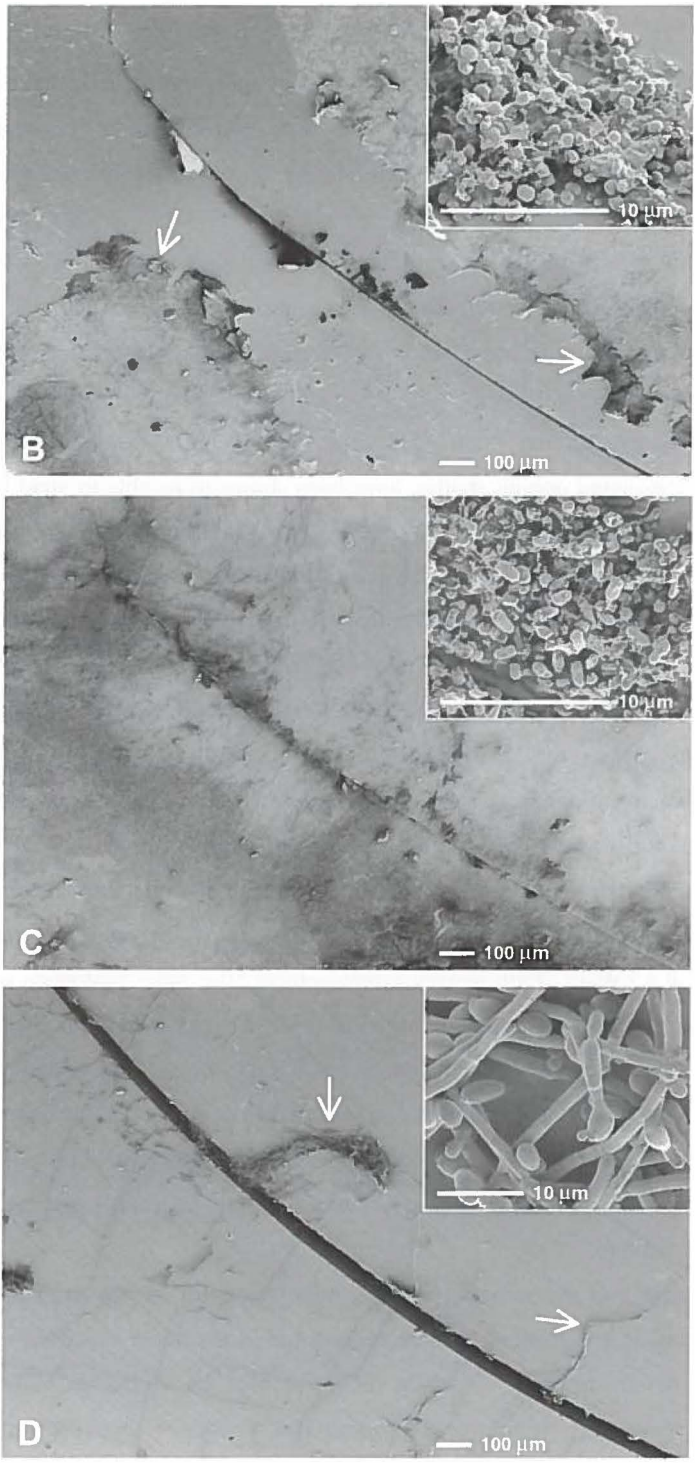


***Figure 4**. Scanning electron micrographs of Groningen button voice prostheses after 7 days biofilm formation in the artificial throat. A)* Streptococcus salivarius *GB 24/9 (clusters of cocci on the valve side are indicated by arrows). B)* Staphylococcus epidermidis *GB 9/6 (patches of biofilm and slime threads are indicated by arrows) C)* Escherichia coli *GBJ 32/28D. D)* Candida albicans I *GBJ 13/4A (mycelia are indicated by arrows).*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Discussion

In the present study, causative bacterial and fungal strains responsible for increases in air flow resistance of silicone rubber voice prostheses have been identified. Conspicuously, voice prosthetic biofilms formed on the esophageal side by *Candida* biofilms did not show the largest increase in air flow resistance. In literature there seems to be agreement that *Candida* species are mainly responsible for deterioration of silicone rubber voice prostheses,[7,11,13,14] which does not necessarily yield failure of a prosthesis. Nevertheless, in order to slow down the colonization of voice prostheses by yeasts, otolaryngologists frequently apply oropharyngeal yeast decontamination by using antifungal agents. In this study, however, the biofilms formed by bacterial strains (*S. aureus* GB 2/1 and *R. dentocariosa* GBJ 41/25B), including their excreted organic matter, have been shown to be able to yield larger increases in air flow resistance (more then 30 cm H_2O.s/l) than biofilms formed by *Candida* species. Note that increases in air flow resistance constitute direct reasons for prosthesis replacement. Therefore, one of the clinical implications of this study is, that prevention of biofilm formation on silicone rubber voice prostheses should also be aimed at reducing the prevalence of these bacterial strains. In 1993, Palmer *et al.*[8] already reported that *S. aureus* acts in concert with *Candida* species and that clinically this cooperation plays a significant role in deterioration of the esophageal surface of voice prostheses. The use of a combined anti-*Candida* and anti-staphylococci drug was suggested to prolong the lifetime of voice prostheses. In addition, Elving *et al.*[23] recently demonstrated that *R. dentocariosa* was more often isolated from explanted prostheses in a patient group requiring replacement within four months than in a patient group requiring replacement after more prolonged periods of time. Consequently, it can be concluded that the identification of *S. aureus* and of *R. dentocariosa* as bacterial strains responsible for increases in air flow resistance corresponds with clinical observations on the prevalences of these strains on failed prostheses. Interestingly, Millsap *et al.*[24] demonstrated that these bacterial strains are specifically able to exert adhesive interactions with yeasts through protein-protein binding[25] or lectin-sugar interactions,[26] and acid-base interactions.[27,28]

Comparison of the scanning electron micrographs of all the oropharyngeal bacterial and yeast strains tested, leads to the general conclusion that more contiguous biofilms yields higher increases in air flow resistance (compare Fig. 4A with Fig. 4C). Moreover, the bacterial strains causing strong increases in air flow resistance seem connected by excreted

organic matter in the form of slime threads. Apparently, these bacterial strains are capable of forming a glycocalix-like structure that eventually closes the valve and that might be disrupted by the prophylactic use of mucolytics. Longterm use of mucolytics is common clinical practice in cystic fibrosis patients and can be done safely, without the danger of inducing any microbial resistance, as antimycotic and antibiotic drugs do.[29]

Summarizing, this study has identified *S. aureus* and *R. dentocariosa* as bacterial strains responsible for increases in air flow resistance of silicone rubber voice prostheses, while in addition glycocalix-like structures have been shown to connect organisms in bacterial biofilms. Consequently, *Candida* species, though responsible for deterioration of the silicone material, might not be the organism causing clinically unacceptable increases in air flow resistance of voice prostheses and the use of mucolytics is suggested as an alternative for antimycotics to prolong the lifetime of voice prostheses.

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Chapter 5

Influence of Different Combinations of Bacteria and Yeasts in Voice Prosthetic Biofilms on the Air Flow Resistance of Prostheses

Elving GJ, Van der Mei HC, Busscher HJ, Van Weissenbruch R, Albers FWJ
Influence of different combinations of bacteria and yeasts in voice prosthetic biofilms on the air flow resistance of prostheses
Antonie van Leeuwenhoek Journal of Microbiology 2001, in press.

Introduction

Silicone rubber voice prostheses, placed in a surgically created fistula in between the trachea and the esophagus, are used to rehabilitate the voice of patients after surgical removal of a malignant tumor in the larynx area, a so-called laryngectomy. After laryngectomy (see Fig. 1), the respiratory tract becomes separated from the digestive tract and when patients intend to speak they close the tracheostoma with a finger to force air through the tracheoesophageal shunt into the esophagus, where remaining muscular structures act as pseudo vocal cords. Normal breathing occurs via the tracheostoma.

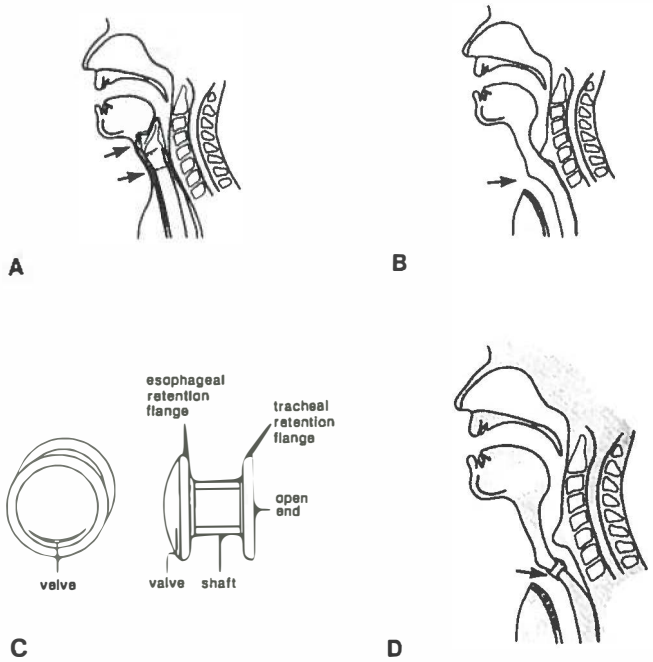


Figure 1. *A) Anatomy of the esophageal region before laryngectomy (arrows indicate parts to be removed). B) Anatomy after laryngectomy showing the separation of the airway and the digestive tract (arrow indicates tracheostoma). C) Sketch of the Groningen button silicone rubber voice prosthesis. D) Groningen button inserted into the tracheoesophageal shunt (arrow).*

Biofilms are considered to be responsible for deterioration of the esophageal surface of voice prostheses causing failure characterized by leakage of liquids through the prosthesis into the trachea or increased air flow resistance. Consequently, replacement of the prosthesis by an otolaryngologist is necessary. Although this procedure is not complicated and can be done in the outpatient department, it is an unpleasant experience for the patient. Moreover, recurrent replacements can cause damage to the tracheoesophageal fistula resulting in external leakage of liquids.[1] Biofilm formation occurs on the entire valve side of a prosthesis, but whether biofilm formation finally leads to dysfunction and replacement of the prosthesis, depends on properties of the biofilm, such as the microbial species in the biofilm and the extension of the biofilm in relation to the valve.

Voice prosthetic biofilms are complex structures consisting of bacteria and yeasts primarily originating from the skin, the oral cavity, food and dairy products. Prevention of biofilm formation is focussed on the fungal component, because yeasts are generally considered to be responsible for deterioration of silicone rubber voice prostheses. This assumption originates from species determination of biofilms from failed prostheses in which *Candida* was present in higher numbers in comparison with other isolated voice prosthetic microorganisms.[2-5] Moreover, examination by light microscopy of the esophageal surface of failed prostheses showed defects in the silicone material caused by yeast-like organisms.[6] As a result of these observations, patients have been put on antimycotic regimes, frequently for extended periods of time, to prolong the lifetime of their voice prostheses. However, there is no convincing evidence that prophylactic application of antimycotics yields an increase in the lifetime of voice prostheses, and this clinical practice likely contributes to the development of antimycotic resistance without any beneficial effects.

Consequently, it would be worthwhile to study the effect of *Candida* species on silicone rubber voice prostheses and examine their influence on the actual dysfunctioning of the valve mechanism. Elving *et al.*[7] determined the effects of single strain biofilms, consisting either of a bacterial or a yeast strain isolated from explanted voice prostheses, on the air flow resistance of silicone rubber voice prostheses. Remarkably, biofilms formed by particular bacterial strains induced larger increases in air flow resistance than biofilms formed by *Candida* species and electron micrographs showed that bacterial biofilms connected by condensed slime threads seemed to block the valve mechanism.

Clinically, colonization of voice prostheses does not involve single strains of either bacteria or yeasts, but both organisms work in concert. This study was undertaken to

determine the effects of combinations of bacterial and yeast strains on air flow resistances of Groningen button voice prostheses in order to identify combinations of bacterial and yeast strains causative to failure of prostheses.

Materials and methods

Voice prostheses

"Low Resistance" silicone rubber Groningen button voice prostheses were kindly provided by Médin Instruments and Supplies (Groningen, The Netherlands). The "Low Resistance" Groningen button voice prosthesis is made of implant grade silicone rubber and consists of a shaft with two flanges with a semicircular slit of 145° in the hat of the esophageal flange, functioning as an one-way valve (Fig. 1C).

Biofilm formation

Groningen button voice prostheses were placed in five modified Robbins devices or artificial throats,[8] made of stainless steel (Fig. 2). Modified Robbins devices were autoclaved before use. Each artificial throat was equipped with two voice prostheses and maintained at temperatures between 36°C and 37°C, as in a laryngectomized patient.

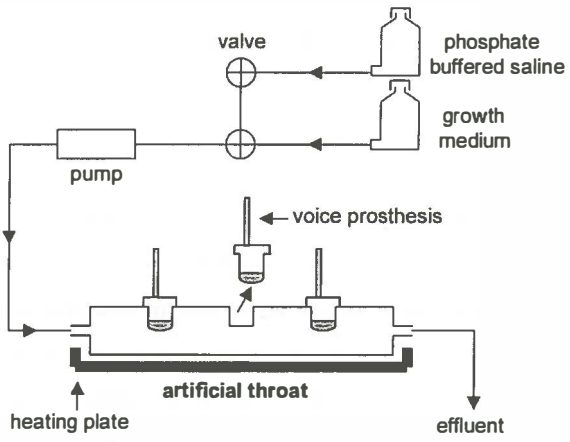


Figure 2. *Schematic presentation of the artificial throat, equipped with three Groningen button voice prostheses.*

Clinically relevant bacterial and yeast strains, previously isolated from explanted Groningen button voice prostheses from different patients and lifetimes varying from 1 to 29 months (see Table 1), were used in various combinations to mimic biofilms as found in laryngectomized patients. Various combinations of strains of bacteria and yeasts were cultured in a mixture of 30% brain heart infusion broth (OXOID, Basingstoke, Great Britain) and 70% defined yeast medium (per litre: 7.5 g glucose, 3.5 g $(NH_4)_2SO_4$, 1.5 g L-asparagine, 10 mg L-histidine, 20 mg DL-methionine, 20 mg DL-tryptophane, 1 g KH_2PO_4, 500 mg $MgSO_4.7H_2O$, 500 mg NaCl, 500 mg $CaCl_2.2H_2O$, 100 mg yeast extract, 500 μg H_3BO_3, 400 μg $ZnSO_4.7H_2O$, 120 μg $Fe(III)Cl_3$, 200 μg $Na_2MoO_4.2H_2O$, 100 μg Kl, 40 μg $CuSO_4.5H_2O$), and used to inoculate the artificial throats during 5 h.

Subsequently, a biofilm was allowed to grow on the voice prostheses during 3 days by filling the devices with growth medium. From day 4 till day 7 the artificial throats were flushed three times a day with 250 ml phosphate buffered saline (10 mmol/l potassium phosphate and 150 mmol/l NaCl, pH 7.0) and left drained. At the end of each day the devices were filled with growth medium during half an hour and subsequently left overnight in the moist environment of the drained throats. The tracheal sides of the prostheses were left in ambient air, similar to the situation with a stoma. Previously, this cycle of feast and famine and exposure to ambient air has been demonstrated essential to grow biofilms with similar features as found on explanted prostheses, as can be demonstrated by comparing Figure 3A, showing heavy biofilm formation on the valve side of an explanted Groningen button voice prosthesis, with Figure 3B showing biofilm formation on a voice prosthesis in the artificial throat.

Measurement of air flow resistances

Compressed air was blown through each voice prosthesis prior to biofilm formation and as covered with a 7 days old biofilm of a given combination of bacterial and yeast strains. Air pressures were varied from 10 to 20 cm H_2O and the resulting air flow (l/s) through the prosthesis was measured using a flow head, calibrated with a Brooks flow meter. The pressure was measured just before the valve of the voice prosthesis with a pressure transducer, calibrated against a water manometer. The pressure range applied corresponds with clinically relevant conditions during tracheoesophageal shunt speech and yielded a linear relationship between air pressure and flow. All prostheses were measured three times and the mean was calculated. From the linear trajectory between air pressure and flow, an air flow resistance

(cm H_2O.s/l) was computed by linear regression analysis, as appropriate for shunt valves.[9] As the air flow resistance of individual voice prostheses prior to biofilm formation differed, the effects of biofilms on the air flow resistance of a prosthesis were expressed relative to the air flow resistance of the same prosthesis prior to biofilm formation.

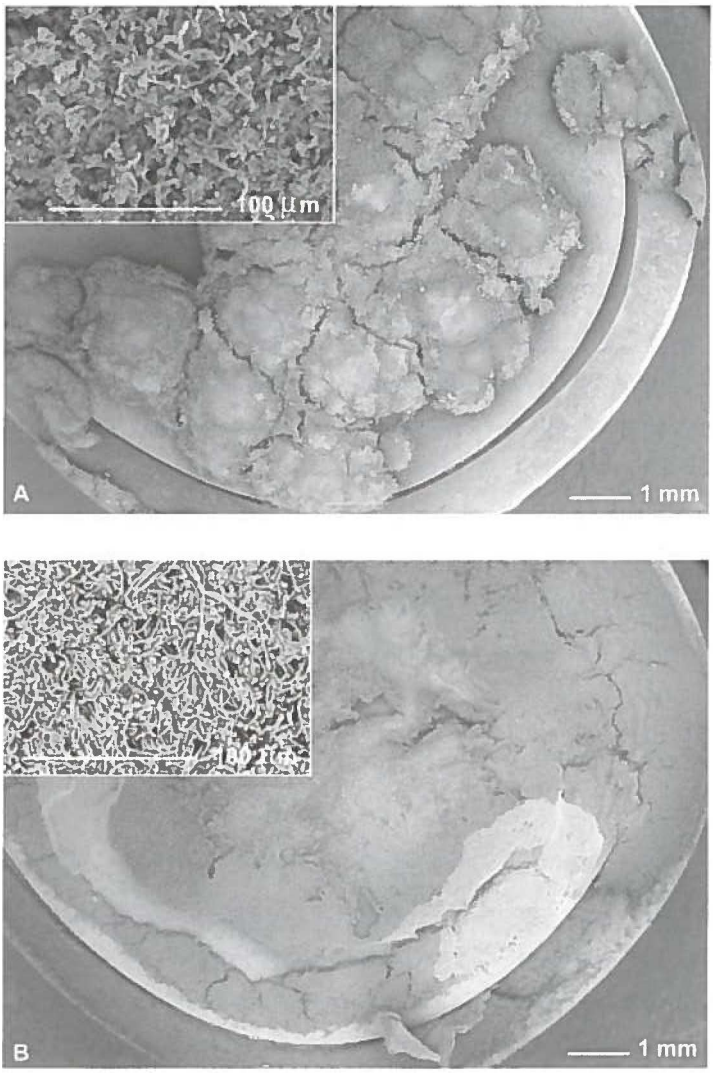


Figure 3. *Scanning electron micrographs of a Groningen button voice prosthesis explanted after 8 months from a laryngectomized patient (A) and after 7 days biofilm formation in the artificial throat (B).*

Evaluation of biofilms

On the eighth day of an experiment, voice prostheses were removed from the artificial throats after a final perfusion with 250 ml phosphate buffered saline. After measuring the air flow resistances of the prostheses, biofilm formation on the valve side of one prosthesis was qualitatively assessed by macroscopic observation with the naked eye and by scanning electron microscopy, while the second prosthesis was used to determine the number of colony forming units (CFU's).

For electron microscopy, biofilm covered voice prostheses were flushed with 6.8% sucrose and 0.1 mol/l cacodylate buffer (pH 7.4), fixed and stained in 2% glutardialdehyde and 0.2% ruthenium red in 0.1 mol/l cacodylate buffer at 4°C and flushed again. Post-fixation and staining was carried out in 1% OsO_4 and 0.2% ruthenium red in cacodylate buffer by gently shaking for 3 h at room temperature. Buffer washes and dehydration involved the following rinsing procedures: 20 min in 6.8% sucrose in 0.1 mol/l cacodylate buffer; 3x10 min bidistilled water; 20 min in respectively 30, 50 and 70% ethanol and 4x30 min in 100% ethanol. After critical-point drying with CO_2 for 4 h, the specimens were mounted on SEM stubs and sputter-coated with gold/paladium (15nm). SEM observations were taken, made using the JEOL 6301, with different magnifications at 15-25 kV.

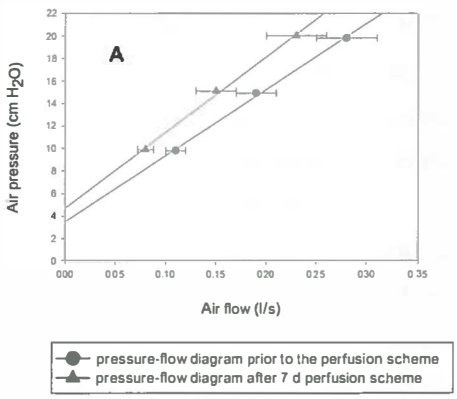
In order to determine the number of CFU's in the biofilm, biofilms were removed by scraping and sonication and subsequently serially diluted. After plating the serial dilution on MRS (de Man, Rogosa and Sharpe) agar plates for yeasts and blood agar plates for bacteria, plates were stored at 37°C in an aerobic incubator for 3 days prior to enumeration.

Results

Air flow resistance

The air flow resistance of the Groningen button voice prostheses used prior to biofilm formation amounted 61 ± 9 cm H_2O.s/l, as averaged over all 90 prostheses involved in this study. The air flow resistance of prostheses increased slightly upon wetting, also in the absence of a biofilm and prostheses placed in the artificial throat after a similar perfusion scheme as during biofilm formation, showed an average increase in air flow resistance of 11 ± 5 cm H_2O.s/l.

Figure 4 shows pressure-flow diagrams of "Low Resistance" Groningen button voice prostheses. Figure 4A demonstrates a pressure-flow diagram prior to and after a 7 days perfusion scheme, in the absence of biofilm (control). Figure 4B represents the relationship between pressure and flow prior to and after 7 days biofilm formation, comprising *C. tropicalis* GB 9/9, *C. albicans* GBJ 13/4A, *S. aureus* GB 2/1, *S. epidermidis* GB 9/6 and *R. dentocariosa* GBJ 52/2B. The air flow resistance increases slightly in the absence of a biofilm, due to the 7 days perfusion scheme, as indicated by the increase in the slope of the lines in Figure 4A. However, the presence of a 7 days old biofilm comprising *C. tropicalis* GB 9/9, *C. albicans* GBJ 13/4A, *S. aureus* GB 2/1, *S. epidermidis* GB 9/6 and *R. dentocariosa* GBJ 52/2B on the esophageal side of the prosthesis yielded much stronger increases in air flow resistance (see Fig. 4B).



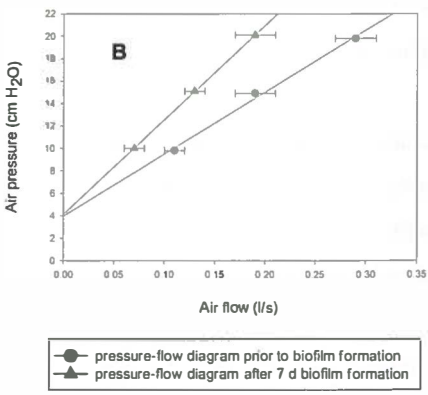


Figure 4. *Examples of pressure-flow diagrams of "Low Resistance" Groningen button voice prostheses.*
A) Prior to and after 7 days perfusion scheme, in the absence of a biofilm. This perfusion scheme is similar as during biofilm formation.
B) Prior to and after 7 days biofilm formation, comprising C. tropicalis *GB 9/9*, C. albicans *GBJ 13/4A*, S. aureus *GB 2/1*, S. epidermidis *GB 9/6 and* R. dentocariosa *GBJ 52/2B, in the artificial throat.*

Table 1 summarizes the increases in air flow resistance of "Low Resistance" Groningen button voice prostheses caused by various 7 days old oropharyngeal biofilms in the artificial throat. All statistically significant (Student *t* test, $p < 0.05$) increases in air flow resistance due to biofilm formation as compared to a control (i.e. a wetted prosthesis without biofilm) are indicated by an asterisk. Voice prosthetic biofilms formed on the esophageal side comprising *C. tropicalis* GB 9/9, *C. albicans* GBJ 13/4A, *S. salivarius* GB 24/9, *S. aureus* GB 2/1, *S. epidermidis* GB 9/6 and *R. dentocariosa* GBJ 52/2B caused the strongest increases in air flow resistance (26 cm H_2O.s/l). Moreover, deletion of *S. salivarius* GB 24/9 or *Staphylococcus* strains from the biofilm yielded even higher increases in air flow resistance (up to 28 cm H_2O.s/l). Alternatively, deletion of *C. tropicalis* GB 9/9 or *R. dentocariosa* GBJ 52/2B caused a smaller increase in air flow resistance of the prostheses by 19 and 15 cm H_2O.s/l, respectively.

Biofilms comprising *C. tropicalis* GB 9/9, *C. albicans* GBJ 13/4A, *S. anginosus* GBJ 27/5C, *R. dentocariosa* GBJ 41/25B, *E. faecalis* 1131 and *S. mucilaginosus* GB 16/3 caused increases in air flow resistance of 19 cm H_2O.s/l. Deletion of *E. faecalis* 1131, *R. dentocariosa* GBJ 41/25B or *S. anginosus* GBJ 27/5C from the biofilm resulted in minor differences in air flow resistance (17 – 14 cm H_2O.s/l).

Voice prosthetic biofilms formed on the esophageal side by the biofilm combinations comprising *C. tropicalis* GB 9/9, *C. albicans* GBJ 13/4A, *S. salivarius* GBJ 52/2A, *S. epidermidis* GB 9/6, *R. dentocariosa* GBJ 52/2B and *E. coli* GBJ 32/28D showed a negligible increase in the voice prosthetic air flow resistance of 10 cm H_2O.s/l. The presence of *R. dentocariosa* GBJ 52/2B in these biofilms did not affect the increase in air flow resistance (10 versus 13 cm H_2O.s/l). In the absence of *S. salivarius* GBJ 52/2A, *S. epidermidis* GB 9/6 or *E. coli* GBJ 32/28D in a biofilm, the increases in voice prosthetic air flow resistances were higher than in their presence (15 - 17 cm H_2O.s/l).

Colony forming units and scanning electron microscopy

Table 1 also summarizes the numbers of yeast and bacterial colony forming units found in the biofilms for the different species combinations employed. Interestingly, no relation exists between the amount of yeasts and bacteria present on a prosthesis and the increases in air flow resistance measured. Macroscopic biofilm formation was only observed twice, namely for the combination of *C. tropicalis* GB 9/9, *C. albicans* GBJ 13/4A, *S. anginosus* GBJ 27/5C, *R. dentocariosa* GBJ 41/25B, *E. faecalis* 1131 and *S. mucilaginosus* GB 16/3 and for the

Table 1. *The number of bacterial and yeast colony forming units per cm^2 on the esophageal surface of "Low Resistance" silicone rubber Groningen button voice prostheses after 7 days biofilm formation in the artificial throat, together with the increases in air flow resistance caused by these biofilms. Scanning electron micrographs were used to evaluate microscopic biofilm formation and ingrowth on the esophageal side of the prosthesis.*

Cambinations of bacteria & yeasts	Candida tropicalis GB 9/9	Candida albicans GBJ 13/4A	Streptococcus salivarius GB 24/9	Streptococcus salivarius GBJ 52/2A	Streptococcus anginosus GBJ 27/5C	Staphylococcus aureus GB 2/1	Staphylococcus epidermidis GB 9/6	Rothia dentocariosa GBJ 52/2B	Rothia dentocariosa GBJ 41/25B	Enterococcus faecalis 1131	Stomatococcus mucilaginosus GB 16/3	Escherichia coli GBJ 32/28D	Increase of air flow resistance (cm H_2O.s/l)	Yeast colony forming units (x 10^6 per cm^2)	Bacterial colony forming units (x 10^6 per cm^2)	Macroscopic biofilm esophageal side prosthesis	Microsopic biofilm esophageal side prosthesis	Microscopic ingrowth esophageal side prosthesis
1	x	x	x					x					28 ± 19*	1.6	10.0	no	yes	no
2	x	x				x	x	x					26 ± 6*	0.3	1.4	no	yes	no
3	x	x	x			x	x	x					26 ± 15*	0.2	0.8	no	yes	yes
4		x	x			x	x	x					19 ± 9	0.5	9.7	no	yes	no
5	x	x			x				x	x	x		19 ± 10	10.8	47.7	yes	yes	no
6	x	x			x				x		x		17 ± 8	4.9	40.6	no	yes	no
7	x	x		x			x	x					17 ± 12	0.9	3.1	no	yes	no
8	x	x	x			x	x						15 ± 3	3.4	16.9	yes	yes	no
9	x	x							x	x	x		15 ± 9	13.2	93.6	no	yes	yes
10	x	x		x				x				x	15 ± 9	1.0	19.4	no	yes	no
11	x	x					x	x				x	15 ± 12	0.5	7.9	no	yes	no
12	x	x			x					x	x		14 ± 5	2.7	8.0	no	yes	no
13	x	x		x			x					x	13 ± 9	1.0	17.4	no	yes	no
14													11 ± 5	0	0	no	no	no
15	x	x		x			x	x				x	10 ± 5	1.1	36.2	no	yes	no

Data are mean values with standard deviations of three separate experiments. Asterisks indicate significant differences (Student t test, $p < 0.05$) from the control (i.e. a wetted prosthesis).

combination of *C. tropicalis* GB 9/9, *C. albicans* GBJ 13/4A, *S. salivarius* GB 24/9, *S. aureus* GB 2/1 and *S. epidermidis* GB 9/6 (Fig. 5C). Note that the macroscopic observation of biofilm does not correlate with the strongest increases in air flow resistance.

All combinations of bacteria and yeasts employed, showed microscopic biofilm formation. Microscopic ingrowth into the esophageal side of the prosthesis was only observed for biofilms comprising *C. tropicalis* GB 9/9, *C. albicans* GBJ 13/4A, *S. salivarius* GB 24/9, *S. aureus* GB 2/1, *S. epidermidis* GB 9/6 and *R. dentocariosa* GBJ 52/2B, or comprising *C. tropicalis* GB 9/9, *C. albicans* GBJ 13/4A, *R. dentocariosa* GBJ 41/25B, *E. faecalis* 1131 and *S. mucilaginosus* GB 16/3 (Fig. 5D). Note that microscopic ingrowth occurs in the absence of a macroscopically visible biofilm.

Figure 5 shows scanning electron micrographs of voice prostheses removed from the artificial throat after 7 days with biofilms of different combinations of bacteria and yeasts. Figure 5A shows the combination of bacteria and yeasts causing the strongest increase in air flow resistance (28 cm H_2O.s/l), comprising *S. salivarius* GB 24/9, *R. dentocariosa* GBJ 52/2B, *C. tropicalis* GB 9/9 and *C. albicans* GBJ 13/4A. The biofilm is formed primarily in and around the valve. Figure 5D shows the biofilm of a combination of bacterial and yeast strains causing only a minor increase in air flow resistance (15 cm H_2O.s/l). This biofilm formed by *E. faecalis* 1131, *S. mucilaginosus* GB 16/3, *R. dentocariosa* GBJ 41/25B, *C. tropicalis* GB 9/9 and *C. albicans* GBJ 13/4A is characterized by ingrowth of biofilm organisms into the silicone rubber equally scattered over the valve side, but leaves the valve itself unaffected. Figure 5B involves a biofilm comprising *S. salivarius* GB 24/9, *S. epidermidis* GB 9/6, *S. aureus* GB 2/1, *R. dentocariosa* GBJ 52/2B, *C. tropicalis* GB 9/9 and *C. albicans* GBJ 13/4A with similarly scattered ingrowth as can be observed in Figure 5D, but with more preferential colonization near the valve. Heavy biofilm formation on the valve side can be seen in Figure 5C, but the major part of the valve itself is uncovered, corresponding with only a minor increase in air flow resistance, as the voice prosthetic biofilm in Figure 5D.

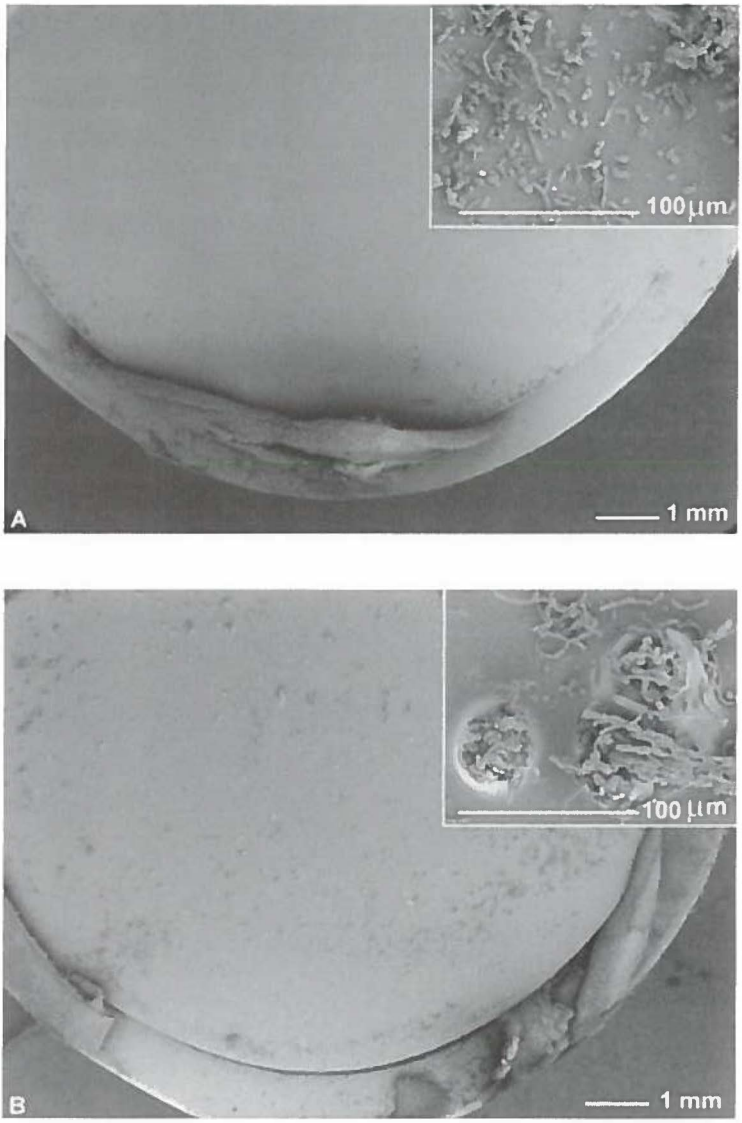


Figure 5. *Scanning electron micrographs of Groningen button voice prostheses after 7 days biofilm formation in the artificial throat. Voice prosthetic biofilms formed on the esophageal side comprising: A)* C. tropicalis *GB 9/9,* C. albicans *GBJ 13/4A,* S. salivarius *GB 24/9 and* R. dentocariosa *GBJ 52/2B. B)* C. tropicalis *GB 9/9,* C. albicans *GBJ 13/4A,* S. salivarius *GB 24/9,* S. aureus *GB 2/1,* S. epidermidis *GB 9/6 and* R. dentocariosa *GBJ 52/2B.*

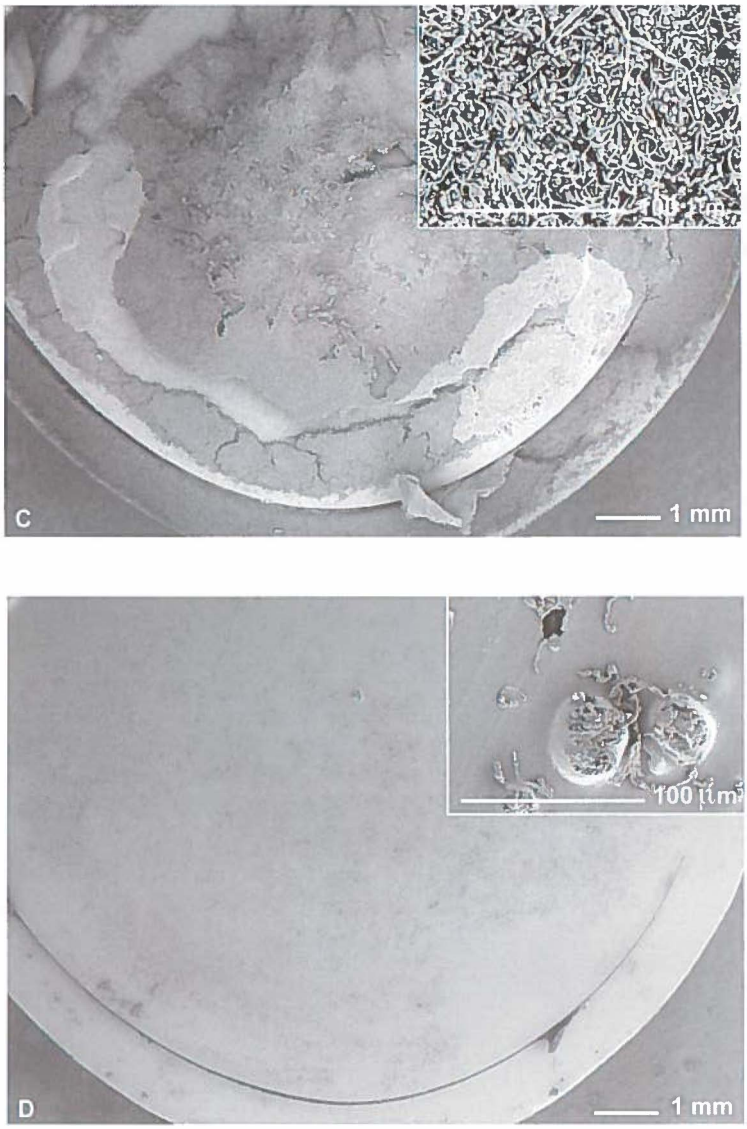


Figure 5 continued. *Scanning electron micrographs of Groningen button voice prostheses after 7 days biofilm formation in the artificial throat. Voice prosthetic biofilms formed on the esophageal side comprising: C)* C. tropicalis *GB 9/9,* C. albicans *GBJ 13/4A,* S. salivarius *GB 24/9,* S. aureus *GB 2/1 and* S. epidermidis *GB 9/6. D)* C. tropicalis *GB 9/9,* C. albicans *GBJ 13/4A,* R. dentocariosa *GBJ 41/25B,* E. faecalis *1131 and* S. mucilaginosus *GB 16/3.*

Discussion

The two major reasons for replacement of silicone rubber voice prostheses are patients complaints about leakage of esophageal contents into the trachea or increasing efforts to produce phonation. These signs of failure of voice prostheses are both due to biofilm formation, a complex microbial structure of organisms comprised in an extensive exopolymeric matrix. The present study identifies causative combinations of voice prosthetic bacterial and yeast strains responsible for increases in air flow resistance of silicone rubber voice prostheses.

Within clinically relevant biofilms causing strong increases in air flow resistances of prostheses (26 to 28 cm H_2O.s/l) and potential clinical failure, the combination of *C. tropicalis*, *S. aureus* and *R. dentocariosa* seems prominent. Deletion of *C. tropicalis* from the biofilm did result in a decrease in air flow resistance from 26 to 19 cm H_2O.s/l, indicating the important role of *C. tropicalis* within this biofilm (see Table 1, compare combination 3 with combination 4). However, the absence of *R. dentocariosa* in the biofilm yielded a much stronger decrease in air flow resistance than deletion of *C. tropicalis* from 26 to 15 cm H_2O.s/l (see Table 1, compare combination 3 with combination 8). These observations are in line with the conclusion by Elving *et al.*[10] that *C. tropicalis* and *R. dentocariosa* play a significant role in the early failure of Groningen button voice prostheses within four months after insertion. Palmer *et al.*[11] suggested that *S. aureus* and *Candida* spp. may act synergistically by stimulating colonization and negatively influencing the valve function. The results of the present study also point to a synergistic cooperation of *S. aureus* and *Candida*, but *R. dentocariosa* coexists in this interaction.

Coexistence of bacteria and yeasts in biofilm formation has been described before, for instance in denture biofilms.[12] Coexistence requires adhesive forces between bacteria and yeasts during their joint colonization of an implant surface and depends among others the surface roughness of the implant, the presence or absence of saliva and the environmental growth conditions in the oral cavity.[13,14] Adhesive forces between bacteria and yeasts have been described biochemically as protein-protein bonds[13] or lectin-sugar interactions,[15] while in physico-chemical terms acid-base interactions[16] and the absence of electrostatic repulsion[17] is of importance for the interference between oropharyngeal bacteria and yeasts. Especially *S. aureus*, *R. dentocariosa*, *S. mucilaginosus* and *Streptococcus mitis* have been shown to stimulate adhesion of *C. albicans* and *C. tropicalis* to biomaterials surfaces in presence of

salivary adhesion mediators *in vitro*.[18] Ell *et al.*[19] found a clinical association between early failure of the voice prostheses because of an increased air flow resistance and *Candida* loading in the esophageal and hinge areas. Moreover, Ell *et al.*[20] demonstrated a relationship between bacterial colony forming units, particularly enterococci, on the esophageal side of the prosthesis and valves failing due to increased resistance within 75 days of use.

Conclusion

For a long time, the presence of yeasts on voice prostheses has been considered causative to valve failure and research has focused on preventing ingrowth. The present study in the artificial throat demonstrates that combinations of bacterial and yeast strains must be identified as causative to voice prosthesis failure rather than a single strain or species.

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Chapter 6

Antimicrobial Activity of Synthetic Salivary Peptides Against Voice Prosthetic Microorganisms

Elving GJ, Van der Mei HC, Busscher HJ, Nieuw Amerongen AV, Veerman ECI,
Van Weissenbruch R, Albers FWJ
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Introduction

Ever since the first total laryngectomy performed by Billroth in 1873, the rehabilitation of the voice of laryngectomized patients has been an important issue to head and neck surgeons. To limit psychological and social problems of postlaryngectomy aphonia, it is essential to succeed in rapid voice restoration. Tracheoesophageal speech by using a voice prosthesis, essentially a shunt-valve between the trachea and the esophagus, is considered to be superior to the other forms of vocal rehabilitation.[1,2] Voice prostheses are usually made of medical-grade silicone rubber, a widely used biomaterial. The nonsterile esophageal environment in which these prostheses are placed, leads to the formation of a mixed biofilm of bacteria and yeasts colonizing these prostheses. Scanning electron microscopy demonstrated that the silicone rubber can be severely deteriorated by ingrowing yeasts, which is generally considered to be the main reason for the frequent valve failure and consequent replacement of voice prostheses.[3] Valve failure mostly becomes evident when patients complain about leakage of liquids or increased efforts to produce tracheoesophageal speech attributable to deterioration and biofilm overgrowth of the valve.

In laryngectomized patients, salivary flow rates are often reduced as a side effect of radiotherapy, while the antimicrobial action of saliva, as a part of the normal host defense, is decreased.[4] To assist the disturbed host defense of laryngectomized patients using voice prostheses, oropharyngeal yeast decontamination by using amphotericin B lozenges and buccal bio-adhesive slow-release tablets containing miconazole nitrate have been attempted without yielding a significant increase in prosthesis lifetime, because the biofilm mode of growth effectively protects the microorganisms against antimycotic agents.[5-7] Moreover, long-term chemoprophylaxis bears the risk of inducing resistant strains, while the number of available antifungal agents is already limited. Surface modification of voice prostheses has been shown to influence biofilm formation in patients, but the impact on prosthesis lifetime remains to be demonstrated. Consumption of dairy products such as buttermilk and Turkish yogurt may also positively affect prosthesis lifetime through reduced biofilm formation.[8]

Histatins, cystatins and lactoferrin are antimicrobial peptides and proteins normally present in saliva with a broad-spectrum antimicrobial activity; lactoferrin is also a major component of buttermilk.[9-11] Until the present, no resistance against these peptides has been reported for any microbial strain. Helmerhorst *et al.*[10,12] demonstrated that both yeast cells and germinated cells of *Candida albicans,* as well as newly emerging non-*C. albicans* strains

including mutants untreatable with currently available antimycotics, are susceptible to natural basic antimicrobial peptides. Based on the pore-formation ability of amphipathic helical peptides, analogues of the C14-terminus of histatin 5 have been developed that differ only slightly in amino acid composition. These synthetic antimicrobial peptides were also effective against planktonic oral bacteria and oral bacteria growing in a biofilm. Recently the antimicrobial activity of these peptides against single cultures of seven oral bacterial strains (*Streptococcus mutans*, *Streptococcus sanguis*, *Streptococcus salivarius*, *Actinomyces naeslundii*, *Veillonella parvula*, *Fusobacterium nucleatum* and *Prevotella intermedia*) was determined and found to be largest for dhvar4, with obligate anaerobic Gram-negative bacteria being more susceptible than facultatively anaerobic or anaerobic bacteria as a whole.[13]

The aim of the present paper is to investigate *in vitro* whether synthetic salivary antimicrobial peptides may have an inhibitory effect on the growth of bacteria and yeasts isolated from used dysfunctional silicone rubber voice prostheses, which not only includes oral bacteria and yeasts but also a large variety of strains from the skin (staphylococci) and other environmental sources.[14]

Materials and methods

Synthetic salivary peptides

Human salivary histatin 5, dhvar1, dhvar4, dhvar5, lactoferrin b 17-30 (LFb 17-30) and cystatin S1-15 (dcysS) were chemically synthesized using the T-bag method as described previously.[10] The amino acid sequence of the peptides can be found in Table 1a and 1b, as derived from Helmerhorst *et al.*[13] All peptides were water soluble and dissolved in 10 mmol/l potassium phosphate (pH 7.0), to final concentrations of 2 mg/ml and 4 mg/ml.

Culture conditions of microorganisms

All microbial strains used (4 yeast and 8 bacterial strains) are listed in Table 2 and were clinical isolates from explanted voice prostheses, except *Enterococcus faecium* 603. Yeast strains were stored on sterile Silicagel 60 (Merck, Darmstadt, Germany) and bacterial strains on plastic beads in TSC vials (Technical Service Consultants, Heywood, Lancs, UK) both at -22°C. *E. faecium* 603 was freeze-dried for storage at -22°C. The microorganisms were grown aerobically in brain heart infusion broth (Oxoid, Basingstoke, Great Britain) at 37°C.

In addition, to investigate whether the synthetic salivary peptides showed activity against a complete total microflora isolated from an explanted voice prosthesis, equal volumes of the single cultured yeasts and bacteria in brain heart infusion broth were mixed. In Table 2 this is defined as "total microflora."

Table 1a. *Amino acid sequence of the antimicrobial peptides.*

Peptide	***Sequence***																						
	1									10										20			
Histatin 5	D	S	H	A	K	R	H	H	G	Y	K	R	K	F	H	E	K	H	H	S	H	R	G	Y
Dhvar1											K	R	L	F	K	E	L	K	F	S	L	R	K	Y
Dhvar4											K	R	L	F	K	K	L	L	F	S	L	R	K	Y
Dhvar5											L	L	L	F	L	L	K	K	R	K	K	R	K	Y
Cystatin S1-15	S	S	S	K	E	E	N	R	I	I	P	G	G	I										

Table 1b. *Amino acid sequence of lactoferrin.*

Peptide	***Sequence***										
	20										30
Lactoferrin b 17-30	F	K	C	R	R	W	Q	W	R	M	K	K	L	G

Microbial growth inhibition test

Yeasts and bacteria cultured overnight under the appropriate conditions were harvested by centrifugation and diluted in reduced transport fluid (NaCl 0.9 g/l, $(NH_4)_2SO_4$ 0.9 g/l, KH_2PO_4 0.45 g/l, $MgSO_4$ 0.19 g/l, K_2HPO_4 0.45 g/l, Na_2 EDTA 0.37 g/l, L-Cysteïne HCl 0.2 g/l, pH 6.8) to a concentration allowing confluent growth when plated with a cotton swab on the agar. Yeasts were plated on MRS agar (De Man, Rogosa and Sharpe, Merck), while bacteria and the total microflora were plated on brain heart infusion agar.

Agar plates were dried for 20 min at room temperature and 5 µl peptide solutions of both the low and high concentration, 2 mg/ml and 4 mg/ml respectively, were spotted onto the

surface of the agar plate. After overnight incubation, the agar plates were screened for growth inhibition zones around the peptide spots. The spectrum of susceptible microorganisms was determined qualitatively. The experiments were scored as positive (+) when growth inhibition was observed; a +/- sign indicated some colonies formed within the zones; and no growth inhibition was marked as negative (-).

Results

Table 2 shows the antimicrobial activities of the synthetic salivary peptides evaluated at two different concentrations against a variety of oropharyngeal microorganisms isolated from explanted voice prostheses.

Histatin 5 showed no antimicrobial activity against the microorganisms involved in this study at either the low or the high concentration. Dhvar1 was active against some of the oropharyngeal microorganisms tested. In the cases of *C. albicans* I GBJ 13/4A and *C. humicola* GBJ 33/9E dhvar1 was active only at the higher concentration tested, while growth of *C. krusei* GBJ 33/9E was not affected. Among the bacterial strains, growth of *R. dentocariosa* GBJ 41/25B, *S. aureus* GB 2/1 and *E. coli* GBJ 32/28D was not inhibited by dhvar1, as was the case with the total microflora.

On the other hand, dhvar4 was active against all oropharyngeal microorganisms involved, including the total microflora. Only at the lower concentration did dhvar4 not show activity against *R. dentocariosa* GBJ 41/25B, *S. aureus* GB 2/1, and the total microflora. Dhvar5 activity was comparable to that of dhvar4, but at the higher concentration dhvar5 did not show activity against *E. coli* GBJ 32/28D and the total microflora.

Lactoferrin b 17-30 (LFb 17-30) did not inhibit the growth of any of the yeast strains tested. Growth of *S. anginosus* GBJ 27/5C and *S. mucilaginosus* GB 16/3 was inhibited at both concentrations of LFb 17-30. Growth of *S. salivarius* GB 24/9, *S. epidermidis* GB 9/6 and *E. faecium* 603 was slightly inhibited at both concentrations, whereas the growth of *R. dentocariosa* GBJ 41/25B and *S. aureus* GB 2/1 was not affected by LFb 17-30. LFb 17-30 inhibited only slightly the growth of the total microflora at the higher concentration tested.

Cystatin S1-15 at both concentrations did not show any antimicrobial activity against the microorganisms involved in this study.

Table 2. *Antimicrobial activity of synthetic salivary peptides at two different concentrations (2mg/ml and 4 mg/ml) against a variety of oropharyngeal microorganisms isolated from explanted voice prostheses.*

	Peptides											
	histatin 5		***dhvar1***		***dhvar4***		***dhvar5***		***lactoferrin b 17-30***		***cystatin S1-15***	
Microorganism	2mg/ml	4 mg/ml	2mg/ml	4 mg/ml	2mg/ml	4 mg/ml	2mg/ml	4 mg/ml	2mg/ml	4 mg/ml	2mg/ml	4 mg/ml
Candida tropicalis GB 9/9	-	-	+	+	+	+	+	+	-	-	-	-
Candida albicans 1 GBJ 13/4A	-	-	-	+	+	+	+	+	-	-	-	-
Candida krusei GBJ 18/4A	-	-	-	-	+	+	+	+	-	-	-	-
Candida humicola GBJ 33/9E	-	-	-	+	+	+	+	+	-	-	-	-
Streptococcus anginosus GBJ 27/5C	-	-	+	+	+	+	+	+	+	+	-	-
Streptococcus salivarius GB 24/9	-	-	+	+	+	+	+	+	+/-	+/-	-	-
Rothia dentocariosa GBJ 41/25B	-	-	-	-	-	+	-	+	-	-	-	-
Staphylococcus aureus GB 2/I	-	-	-	-	-	+	+/-	+	-	-	-	-
Staphylococcus epidermidis GB 9/6	-	-	+	+	+	+	+	+	+/-	+/-	-	-
Stomatococcus mucilaginosus GB 16/3	-	-	+	+	+	+	+	+	+	+	-	-
Escherichia coli GBJ 32/28D	-	-	-	-	+	+	-	-	-	+/-	-	-
Enterococcus faecium 603	-	-	+	+	+	+	+	+	+/-	+/-	-	-
Total cultivable microflora	-	-	-	-	-	+	-	-	-	+/-	-	-

Discussion

In the present study, the antimicrobial activity of different synthetic salivary peptides derived from histatin against a variety of oropharyngeal microorganisms from explanted voice prostheses was investigated. Only dhvar4 has been shown to be able to inhibit the growth of all the separate oropharyngeal microorganisms tested, even when these are acting "in concert" (total microflora). The microorganisms in the present study are thought to be representative of those generally isolated from explanted dysfunctional voice prostheses,[14] although, because of varying endogeneous and exogeneous factors, the oropharyngeal microflora may vary greatly over time. There seems to be agreement in the literature that *Candida* species are mainly responsible for the deterioration of silicone rubber voice prostheses, although adhesion of bacteria has been suggested to be a prerequisite for colonization of voice prostheses by yeasts,[3] similar to the situation in denture stomatitis.[15] However, enterococci have been associated with valve failure attributable to increased resistance, while streptococci have been associated with valves failing as a result of leakage.[16]

Dhvar4 and dhvar5 are the only synthetic peptides with an antimicrobial spectrum broad enough to cover the variety of oropharyngeal microorganisms found on voice prostheses. Interestingly, dhvar5 shows no activity against *E. coli* GBJ 31/28D, whereas, concurrently, its activity against the total microflora, consisting of all organisms that were used acting in concert, is lost. The amino acid sequences of dhvar4 and dhvar5 are entirely different (see Table 1a), whereas the amino acid sequence of dhvar1 differs from that of dhvar4 by only two amino acids (see Table 1a), which leads to a loss of activity of a number of oropharyngeal organisms (see Table 2).

Conclusion

This study shows the potential of using synthetic peptides against oropharyngeal biofilms on voice prostheses. Different pathways can be followed from this investigation for clinical application of the synthetic peptide dhvar4, including oral administration and administration through slow-release mucoadhesive polymers or by coupling the peptide to the silicone rubber itself.

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Chapter 7

The Influence of Antimicrobial Peptides and Mucolytics on the Integrity of Biofilms Consisting of Bacteria and Yeasts as Affecting Voice Prosthetic Air Flow Resistances

Elving GJ, Stokroos I, Nieuw Amerongen AV, Van der Mei HC, Busscher HJ, Van Weissenbruch R, Albers FWJ

The influence of antimicrobial peptides and mucolytics on the integrity of biofilms consisting of bacteria and yeasts as affecting voice prosthetic air flow resistances

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Introduction

Biofilms consisting of a combination of bacterial and yeast strains occur in a number of biomaterials-associated infections in the human body, such as denture stomatitis,[1] biofilms on silicone rubber voice prostheses[2] and on infected nasogastric[3] and endotracheal tubes.[4] Biofilms, however, not only consist of microorganisms, but since the introduction of the confocal scanning laser microscope it is known that most of the volume within a biofilm is devoid of organisms and filled with water or extracellular polymeric substances (EPS).[5] EPS are regarded as the glue, ensuring the integrity of a biofilm. The EPS embedding organisms in a biofilm on biomaterials implants are generally held responsible for the poor influence antibiotics or antimycotics have on biofilm infections and the lack of impact the host immune system has on biomaterials-associated infections. Consequently, infected biomaterials implants often have to be removed[6,7] at high costs and at the expense of patients comfort.

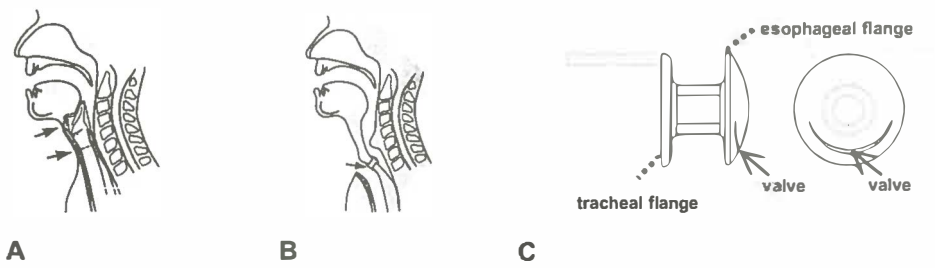


Figure 1. *A) Anatomy of the esophageal region before laryngectomy (arrows indicate parts to be removed). B) Anatomy after laryngectomy showing the separation of the airway and the digestive tract. The arrow indicates a voice prosthesis inserted into the tracheoesophageal shunt, preventing leakage of esophageal contents into the trachea. By closing the tracheostoma with a finger the laryngectomized patient can force air through the valve into the upper digestive tract where remaining muscular structures act as pseudo vocal cords making speech possible. C) Schematic presentation of the "Low Resistance" Groningen button silicone rubber voice prosthesis.*

The integrity of biofilms on voice prostheses, used to rehabilitate speech in patients after laryngectomy (Fig. 1A and 1B),[8] is obligatory to increase the air flow resistance of the valve mechanism of these prostheses. As a result, increasing efforts have to be made by the patient in order to produce speech.[9] Remarkably, explanted malfunctioning voice prostheses with similar lifetimes show enormous variations in biofilm formation on the valve side of a prosthesis varying from a single small colony to a thick biofilm covering the entire valve side. Elving *et al.*[10] suggested that the thickness of biofilms is not the most important issue in valve failure, but rather the combined presence of EPS-producing bacterial strains and yeast species. Scanning electron micrographs of single strain biofilms on voice prostheses, grown in a modified Robbins device, showed massive patches of biofilm in which organisms seem connected by excreted organic matter in the form of slime threads.[11]

Biofilm formation on voice prostheses is influenced by different individual and disease-related factors, such as nutrition, prosthetic tooth replacement, drug therapy, irradiation dose, volume of irradiated salivary gland tissue, residual salivary flow rate and time passed after irradiation.[12,13] Moreover, decreased salivary secretion as a side-effect of radiation therapy reduces the histatin level in the oral cavity, which particularly diminishes the antifungal activity of the saliva.[14] Recently, Elving *et al.*[15] suggested that the oral defense mechanisms in laryngectomized patients might be restored using synthetic salivary peptides to affect the integrity of voice prosthetic biofilms and increase the lifetime of voice prostheses. Opposite to antimycotics, no resistance has been reported against these antimicrobial peptides.[16] Alternatively, since EPS contribute most to the integrity of voice prosthetic biofilms, also mucolytics as used in cystic fibrosis patients may contribute to increasing the lifetime of voice prostheses.[17]

The aim of the present study is to evaluate whether synthetic salivary antimicrobial peptides added to a salivary substitute or mucolytics influence the integrity of biofilms, consisting of bacteria and yeasts, as affecting the air flow resistance of voice prostheses. Two antiseptics, chlorhexidine and triclosan were included as positive controls, while all results are expressed relative to phosphate buffered saline as a negative control.

Materials and methods

Voice prostheses

"Low Resistance" silicone rubber Groningen button voice prostheses were supplied by Médin Instruments and Supplies (Groningen, The Netherlands). The "Low Resistance" Groningen button voice prosthesis (Fig. 1C) consists of a shaft with two flanges with a semicircular slit of 145° in the hat of the esophageal flange, functioning as an one-way valve. The prosthesis is made of implant grade silicone rubber.

Biofilm formation

Groningen button voice prostheses were placed in five modified Robbins devices or artificial throats,[18] made of stainless steel (Fig. 2). Modified Robbins devices were autoclaved before use. Each artificial throat was equipped with two voice prostheses and maintained at temperatures between 36°C and 37°C, as in a laryngectomized patient.

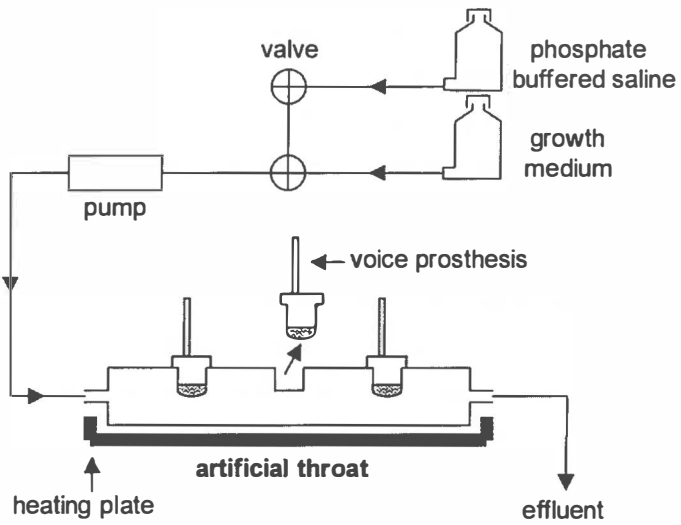


Figure 2. *Schematic presentation of the modified Robbins device, used as an artificial throat, equipped with three Groningen button voice prostheses.*

To mimic biofilms as found in laryngectomized patients, artificial throats were inoculated for 5 h with a combination of bacteria and yeasts, previously isolated from explanted Groningen button voice prostheses from different patients and lifetimes varying from 1 to 29 months. This combination comprised *Candida tropicalis* GB 9/9, *Candida albicans* GBJ 13/4A, *Staphylococcus aureus* GB 2/1, *Staphylococcus epidermidis* GB 9/6, *Streptococcus salivarius* GB 24/9 and *Rothia dentocariosa* GBJ 52/2B,[10] and was cultured in a mixture of 30% brain heart infusion broth (OXOID, Basingstoke, Great Britain) and 70% defined yeast medium (per liter: 7.5 g glucose, 3.5 g $(NH_4)_2SO_4$, 1.5 g L-asparagine, 10 mg L-histidine, 20 mg DL-methionine, 20 mg DL-tryptophane, 1 g KH_2PO_4, 500 mg $MgSO_4.7H_2O$, 500 mg NaCl, 500 mg $CaCl_2.2H_2O$, 100 mg yeast extract, 500 µg H_3BO_3, 400 µg $ZnSO_4.7H_2O$, 120 µg $Fe(III)Cl_3$, 200 µg $Na_2MoO_4.2H_2O$, 100 µg KI, 40 µg $CuSO_4.5H_2O$). After inoculation, a biofilm was allowed to grow on the voice prostheses during 3 days by filling the devices with growth medium. From day 4 till day 7 the artificial throats were perfused three times a day with 250 ml phosphate buffered saline (10 mmol/l potassium phosphate and 150 mmol/l NaCl, pH 7.0). After each perfusion with phosphate buffered saline, the esophageal side of the two voice prostheses of one artificial throat were dipped in phosphate buffered saline, pH 7.0, which served as a negative control, or a salivary substitute with or without synthetic salivary peptides added. Two antiseptic solutions were included as positive controls. In case of mucolytics, the device was flushed with the agent dissolved in sterile water. Subsequently, the prostheses were left in the moist environment of the artificial throats. At the end of each day the devices were filled with growth medium during half an hour and left overnight in the moist environment of the drained artificial throats. The tracheal sides of the prostheses were left in ambient air, similar to the situation with a stoma. Previously, this cycle of feast and famine and exposure to ambient air has been demonstrated to be essential to grow biofilms with features that cannot be distinguished from *in vivo* biodeterioration seen on explanted prostheses.[18] All experiments were carried out in triplicate.

Salivary substitutes, antimicrobial mouthrinses and mucolytics

The salivary substitutes used in this study are Saliva Orthana (Pharmachemie BV, Haarlem, The Netherlands), Glandosane (cell pharm GmbH, Hannover, Germany) and Xialine (Lommerse Pharma, Oss, The Netherlands). Two antiseptics were included as a positive control, viz. chlorhexidine digluconate 0.1% and 0.15% triclosan (Duchefa, Haarlem, The Netherlands), solubilized in propylene glycol (Genfarma BV, Maarssen, The Netherlands).[19]

The mucolytic agent used in this experiment is N-acetylcysteine (Zambon Nederland BV, Amersfoort, The Netherlands), i.e. 400 mg N-acetylcysteine dissolved in 100 ml sterile water.

Synthetic salivary peptides

Dhvar4 and dhvar5 were chemically synthesized using the T-bag method as described previously.[20] The amino acid sequence of the peptides is given in Table 1.[21] Both peptides are water-soluble and were dissolved in Xialine to a final concentration of 4 mg/ml.[22]

Table 1. *Amino acid sequence of the antimicrobial salivary peptides.*

Peptide	***Sequence***													
	10									20				
Dh-5*	K	R	K	F	H	E	K	H	H	S	H	R	G	Y
Dhvar4	K	R	L	F	K	K	L	L	F	S	L	R	K	Y
Dhvar5	L	L	L	F	L	L	K	K	R	K	K	R	K	Y

**The carboxyl terminus of the natural histatin 5.*[20]

Measurement of air flow resistances

Compressed air was blown through each voice prosthesis prior to biofilm formation and as covered with the 7 days old biofilm. Air pressures were varied from 10 to 20 cm H_2O and the resulting air flow (l/s) through the prosthesis was measured using a flow head, calibrated with a Brooks flow meter. The pressure was measured just before the valve of the voice prosthesis with a pressure transducer, calibrated against a water manometer. The pressure range applied corresponds with clinically relevant conditions during tracheoesophageal shunt speech and yielded a linear relationship between air pressure and flow. All prostheses were measured three times and the mean was calculated. From the linear trajectory between air pressure and flow, an air flow resistance (cm H_2O.s/l) was computed by linear regression analysis, as appropriate for shunt valves.[23] In this study the differences in air flow resistance prior to and after 7 days biofilm formation were used to quantify the integrity of the biofilm and the coherency between bacteria and yeasts, as connected by their excreted organic matter. As the air flow resistance of individual voice prostheses prior to biofilm formation differed due to manufacturing, all air flow resistances measured were expressed relative to the air flow resistance of the same prosthesis prior to biofilm formation.

Evaluation of biofilms

On the eighth day of an experiment, voice prostheses were removed from the artificial throats after a final perfusion with 250 ml phosphate buffered saline. After measuring the air flow resistances of the prostheses, biofilm formation on the valve side of one prosthesis was qualitatively assessed by scanning electron microscopy (SEM), while the second prosthesis was used to determine the number of colony forming units (CFU's).

For electron microscopy, biofilm covered voice prostheses were flushed with 6.8% sucrose and 0.1 mol/l cacodylate buffer (pH 7.4), fixed and stained in 2% glutardialdehyde and 0.2% ruthenium red in 0.1 mol/l cacodylate buffer at 4°C and flushed again. Post-fixation and staining was carried out in 1% OsO_4 and 0.2% ruthenium red in cacodylate buffer by gently shaking for 3 h at room temperature. Buffer washes and dehydration involved the following rinsing procedures: 20 min in 6.8% sucrose in 0.1 mol/l cacodylate buffer; 3x10 min bidistilled water; 20 min in respectively 30, 50 and 70% ethanol and 4x30 min in 100% ethanol. After critical-point drying with CO_2 for 4 h, the specimens were mounted on SEM stubs and sputter-coated with gold/paladium (15nm). SEM observations were taken, made using the JEOL 6301, with different magnifications at 15-25 kV.

In order to determine the number of CFU's in the biofilm, biofilms were removed by scraping and sonication and subsequently serially diluted. After plating the serial dilution on MRS (de Man, Rogosa and Sharpe) agar plates for yeasts and blood agar plates for bacteria, plates were stored at 37°C in an aerobic incubator for 3 days prior to enumeration. The number of bacterial and yeast colony forming units on the esophageal surface of each prosthesis was determined separately and expressed as a percentage with respect to the control. The consistency of the biofilm formation in each run was secured by comparison with the control throat.

Statistical analysis

All data were compared with respect to the control, a paired Student t test was used for the statistical analysis, and accepting $p < 0.1$ as statistically significant.

Results

Figure 3 shows scanning electron micrographs of voice prostheses removed from the artificial throat after 7 days of biofilm formation as influenced by the different chemicals evaluated. The biofilm covering the valve side of prostheses removed from the control throat is characterized by the presence of microbial communities of bacteria and yeasts adhering closely together with condensed EPS and connecting slime threads (Fig. 3A). The effect of N-acetylcysteine on biofilm formation is shown in Figure 3B. Although communities of bacteria and yeasts still exist, condensed EPS and connecting slime threads are absent, corresponding with a significant decrease in air flow resistance, as compared with the control (see Table 2).

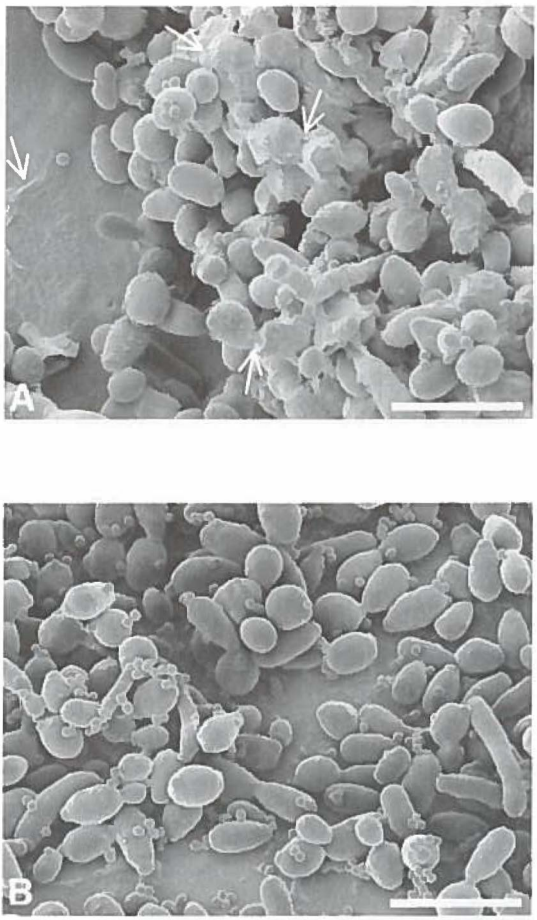


Figure 3. *Scanning electron micrographs of Groningen button voice prostheses after 7 days biofilm formation influenced by different chemicals.*
The bars represent 10 µm.
A) Phosphate buffered saline, negative control (patches of slime and slime threads are indicated by arrows)
B) N-acetylcysteine

In Figure 3C it can be seen that 0.15% triclosan decreases the prevalence of bacteria in the biofilm, which becomes dominated by yeast hyphae. Also these biofilms show a significant decrease in air flow resistance, as compared with the control (Table 2). Chlorhexidine digluconate 0.1% seems to eliminate nearly all bacteria from the biofilm, while it leaves malformed yeasts (Fig. 3D). Xialine with dhvar5 added has very little effect on the appearance of the biofilms (Fig. 3E), while condensed EPS and connecting slime threads are clearly visible, as on the control (see Fig. 3A). Xialine with dhvar4 added causes destruction of yeast hyphae (Fig. 3F) constituting a web-like construction upon the valve side. Below this web bacteria are visible, attached to the valve side. This is concurrent with an increase in air flow resistance (see Table 2).

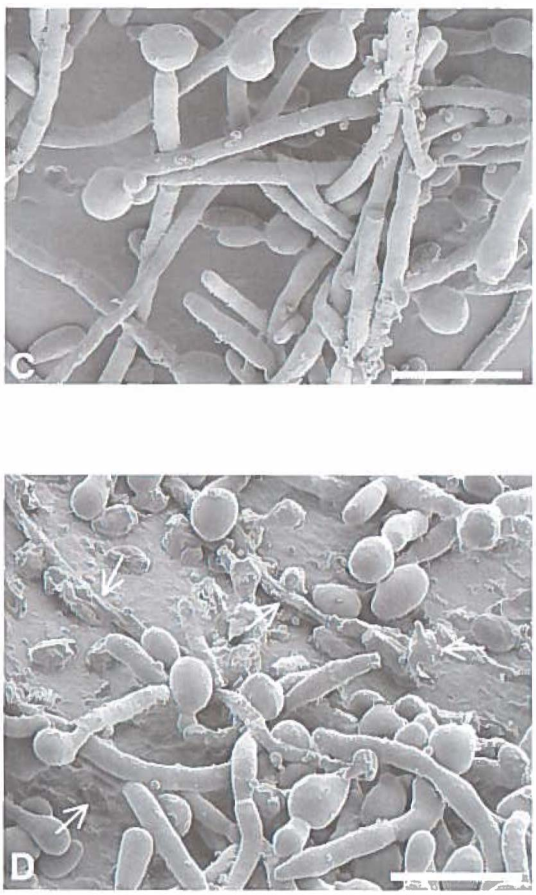
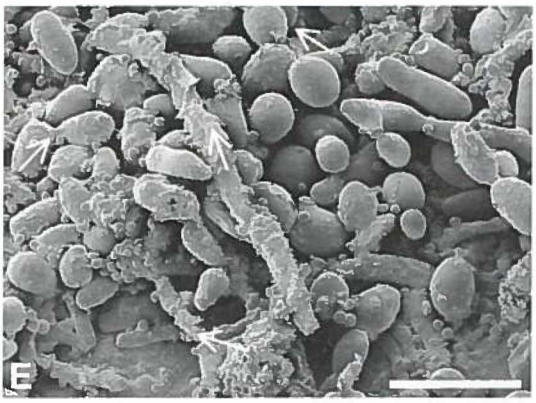


Figure 3 continued. *Scanning electron micrographs of Groningen button voice prostheses after 7 days biofilm formation influenced by different chemicals.*
The bars represent 10 μm.
C) Triclosan 0.15%
D) Chlorhexidine digluconate 0.1%
(malformed yeasts are indicated by arrows)

Figure 3 continued. *Scanning electron micrographs of Groningen button voice prostheses after 7 days biofilm formation influenced by different chemicals.*
The bars represent 10 µm.
E) Xialine with dhvar5 (4 mg/ml) added (slime threads are indicated by arrows)
F) Xialine with dhvar4 (4 mg/ml) added (destructed yeast hyphae are indicated by arrows)



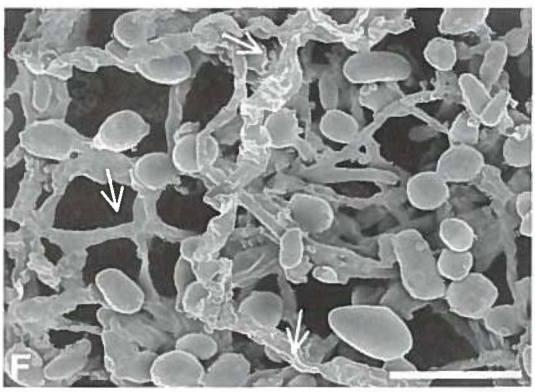


Table 2 summarizes the percentage of bacteria and yeasts on the esophageal side of Groningen button voice prostheses. Both N-acetylcysteine and triclosan 0.15% showed a significant decrease of the amount of bacteria in the biofilm to 28% and 8% of the control, respectively. Also, yeast prevalence was diminished in biofilms affected by N-acetylcysteine and triclosan 0.15% as compared to the control. Chlorhexidine digluconate 0.1% reduced the amount of bacteria in the biofilm to 2% and the amount of yeasts to 6% of the control. Saliva Orthana did not significantly alter the microbial composition of the voice prosthetic biofilms, while Xialine and Glandosane tripled yeast prevalence in the biofilms. Glandosane also triples

the amount of bacteria, but Xialine yielded a small reduction in bacterial prevalence (77% of the control). Addition of dhvar5 to Xialine yielded decreases in both the percentages of bacteria (22% of the control) and yeasts (6% of the control). Xialine with dhvar4 added did hardly affect the amount of bacteria and the amount of yeasts showed a small increase compared to the control.

Table 2. *Decreases (-) and increases (+) in air flow resistance caused by biofilms influenced by salivary substitutes with or without synthetic salivary peptides added, an antimicrobial mouthrinse or a mucolytic compared with the effects of phosphate buffered saline as a control. The relative increase in air flow resistance caused by the control was set at 0 cm H_2O.s/l. Also included are the percentage of viable bacteria and yeasts isolated from the voice prostheses after biofilm formation in the artificial throat. Both for bacteria and yeasts, the number of organisms found after using phosphate buffered saline as a control was set at 100%. All experiments were carried out in triplicate.*

Agents tested	*Percentage of bacteria*[a,b]	*Percentage of yeasts*[a,b]	*Decrease (-) / increase (+) in air flow resistance (cm H_2O.s/l)*[b]
N-acetylcysteine	28*	37**	-34 ± 36*
Triclosan 0.15%	8*	6**	-30 ± 36*
Chlorhexidine digluconate 0.1%	2**	6**	-12 ± 18
Saliva Orthana	113	91	-3 ±13
Phosphate buffered saline	**100**[c]	**100**[c]	**0**[d]
Xialine	77	330	+0.4 ± 36
Glandosane	384	307*	+6 ± 27
Xialine/dhvar5 (4 mg/ml)	22	6	+8 ± 9*
Xialine/dhvar4 (4 mg/ml)	112	144	+63 ± 94**

[a] *Standard deviation over three experiments amounts 20-30%.*

[b] *A single asterisk (*) indicates significant differences (paired Student t test, $p < 0.1$) from the control, while ± indicate standard deviations. Double asterisks (**) indicate significant differences (paired Student t test) in case accepting $p < 0.15$ as statistically significant.*

[c] *The number of bacterial and yeast colony forming units of the control amounts respectively 4.5×10^7 and 9.1×10^6 per cm^2 on the esophageal surface of the "Low Resistance" Groningen button voice prosthesis.*

[d] *Phosphate buffered saline causes an increase in air flow resistance of 29 ± 29 cm H_2O.s/l.*

Table 2 also shows the effects on the air flow resistance of biofilms as influenced by the different chemicals evaluated, expressed relative to effects of phosphate buffered saline. The air flow resistance of the Groningen button voice prostheses used prior to biofilm formation amounted 71 ± 7 cm H_2O.s/l, as averaged over all 62 prostheses involved in this study. The air flow resistance of prostheses increased on average by 29 ± 29 cm H_2O.s/l after 7 days biofilm formation and perfusion of the artificial throats with phosphate buffered saline (negative control). N-acetylcysteine and triclosan 0.15% caused a significant decrease in air flow resistance of 34 and 30 cm H_2O.s/l respectively, as compared with the control. Small, but statistically insignificant changes in air flow resistances were caused by chlorhexidine digluconate 0.1% and the three salivary substitutes (Saliva Orthana, Xialine and Glandosane). Addition of dhvar5 to Xialine showed a small, but significant increase in air flow resistance of 8 cm H_2O.s/l with respect to the control. Unexpectedly, Xialine/dhvar4 yielded a strong increase in air flow resistance of 63 cm H_2O.s/l, compared with the control.

Discussion

In this study, alternatives have been evaluated for antifungal agents currently applied by otolaryngologists in case of recurrent candidiasis in laryngectomized patients that cause malfunctioning of voice prostheses.[24] The synthetic histatin analogues dhvar4 and dhvar5 have been included because of their attractive broad antimicrobial activity *in vitro* against a variety of voice prosthetic oropharyngeal microorganisms.[15] Moreover, until now no microbial resistance against these salivary peptides has been reported. This item is of great importance since laryngectomized patients with tracheoesophageal speech by using a voice prosthesis are mainly benefited by frequent and long-term "anti-biofilm" therapy. Helmerhorst *et al.*[21] determined *in vitro* the antimicrobial activity of the synthetic salivary peptide dhvar4 against a biofilm formed on hydroxyapatite discs consisting of seven oral bacterial species in the absence of yeasts. Dhvar4 showed to be capable of significantly reducing all bacterial strains within the biofilm. Additionally, Helmerhorst *et al.*[20] demonstrated the candidacidal activity of histatin analogues by growth inhibition on agar, but the effects of histatin analogues on oral yeast strains employing a biofilm model have never been evaluated.

Interestingly, in this study biofilms consisting of bacteria and yeasts offered full protection against dhvar4, while dhvar5 was effective in reducing the number of bacteria and

yeasts in mixed species biofilms. Unfortunately, this reduction was not accompanied by a reduction in air flow resistance, suggesting that the integrity of the biofilm was not affected. Figure 3E suggests that this is due to EPS and connecting slime threads still being present in the biofilm.

The three salivary substitutes evaluated showed similar effects on air flow resistance differing slightly from the control. Saliva Orthana had no influence on the microbial composition of the biofilms, but Xialine and Glandosane both stimulated the presence of yeasts in the biofilms. Possibly, yeast grow on nutrients, provided by bacterial degradation products of xanthan gum and carboxymethylcellulose, ingredients of Xialine and Glandosane, respectively. In addition, Glandosane also stimulated bacterial prevalence in the biofilms. Despite the fact that Glandosane and Xialine stimulated microbial growth in the voice prosthetic biofilms, no significant effects on air flow resistances were observed, indicating that microbial prevalence in biofilms is not the most determinant factor controlling the air flow resistance of prostheses.

Chlorhexidine digluconate 0.1% and triclosan 0.15%, both antiseptics used in mouthrinses, were included as positive controls in this study and both chemicals decreased microbial prevalence in the biofilms, with concurrent decreases in air flow resistance, most significantly by triclosan. Chlorhexidine digluconate 0.1%, however, is not appropriate for long-term use, because of irritation and dehydration of the mucosa and staining of teeth or prosthetic replacements. Triclosan is manufactured for use in oral preparations and is incorporated in toothpastes to fight cavities, plaque and gingivitis.[25,26] Unfortunately, the first resistant strains against triclosan have already been reported.[27]

Based on the above, it can be concluded that the integrity of voice prosthetic biofilms is not determined by the number of organisms in the biofilm, but much more by EPS production, glueing the biofilm together and impacting the air flow resistance of voice prostheses. This is confirmed by the observation that the mucolytic N-acetylcysteine, opening di-sulfide bridges between EPS macromolecules and directly interfering with bacterial EPS production mechanisms, induced a significant decrease in air flow resistance while only slightly affecting microbial prevalence. Perez-Giraldo *et al.*[28] studied the influence of various concentrations of N-acetylcysteine on the formation of biofilms of different strains of *S. epidermidis* and found a dose-related decrease in biofilm and slime formation. N-acetylcysteine therefore is a promising chemical to disrupt the integrity of voice prosthetic biofilms, especially since it can be swallowed and used longtime without adverse effects. It is

therefore surprising that N-acetylcysteine has not been used more often in prosthetic voice restoration, either to prevent biofilm formation and the occurrence of increased air flow resistance or for the repair of malfunctioning prostheses.

In summary, this study shows the importance of EPS in maintaining the integrity of biofilms on voice prostheses, as affecting their air flow resistances. Apparently, it is more effective to decrease the integrity of biofilms through direct effects on the EPS than to reduce the number of viable organisms on the esophageal surface of voice prostheses. Moreover, mucolytics are more suitable for frequent and long-term use than antimicrobial agents, as they do not induce microbial resistance nor cause harmful side-effects in patients.

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Chapter 8

General Discussion

Different methods for restoring the lost voice of laryngectomized patients have been developed. The insertion of a silicone rubber voice prosthesis in a surgically created tracheoesophageal shunt or fistula was a major step forward in the speech rehabilitation of laryngectomized patients and is now generally considered to be superior to any other form of substitute voice production. Voice prostheses are not permanent implants, but need to be replaced when patients complain about leakage through or around the prosthesis, or increased efforts to produce tracheoesophageal speech. Continuous exposure to saliva, food, drinks, and the oropharyngeal microflora contributes to the rapid colonization by a mixed biofilm of bacteria and yeasts, leading to valve failure and frequent exchange of the implant.

In this thesis, biofilms on silicone rubber voice prostheses, generally held responsible for malfunctioning of the valve mechanism, were analyzed both *in vivo* and *in vitro*. On the basis of *in vivo* results, acquired by identifying microorganisms isolated from explanted voice prostheses from different patients and lifetimes, a method was developed to grow biofilms, as found in laryngectomized patients, under laboratory conditions. Such biofilms under *in vivo* conditions most frequently consisted of combinations of 4 or more strains and were grown under *in vitro* conditions out of about 4 to 6 microbial strains. However, it is also of importance to be acquainted with the properties of single microbial strains once attached to the silicone rubber valve side of voice prostheses. The combined studies led us to define particular combinations of yeast and bacterial strains, causative for the limited lifetimes of silicone rubber voice prostheses.

A clinically most relevant aspect of this thesis is the prevention of biofilm formation on voice prostheses, affecting the valve mechanism. Extracellular polymeric substances in the form of slime threads excreted by some voice prosthetic microorganisms were identified as a glue, ensuring the integrity of the biofilm and therewith causing valve failure. Based on the results of this thesis, mucolytics are advocated for clinical use in order to prolong the lifetime of silicone rubber voice prostheses.

Voice prostheses

In Western Europe, the self-retaining Low Resistance voice prostheses, such as the Provox 2 and the Groningen button voice prostheses, are frequently applied. The Provox 2 voice prosthesis is nowadays more frequently applied compared to the Groningen button voice prosthesis as the anterograde replacement method is preferred by patients. However, for

research purposes the Low Resistance Groningen button voice prosthesis was chosen for its design features. The valve of the Groningen button voice prosthesis as well as the Provox 2 voice prosthesis is made of silicone rubber. The design of the valve differs, since the Low Resistance Groningen button voice prosthesis has a semicircular slit valve in the hat of the esophageal flange, whereas the Provox 2 voice prosthesis is constituted of a recessed hinged valve. However, to study biofilm formation the large esophageal valve surface of the Groningen button voice prosthesis is more easily accessible than the valve of the Provox voice prosthesis which is not in level with the surrounding esophageal flange.

In this thesis the integrity of the biofilm was chosen as dimension for failure of the valve mechanism, therefore the air flow resistance of a prosthesis with biofilm formation was expressed relative to the air flow resistance of the same prosthesis prior to biofilm formation to be able to quantify which biofilm composition with its excreted organic matter provokes the most serious dysfunction of the valve mechanism. It can be assumed, that this method to quantify valve failure could be used for various valve designs, the slit valve of the Groningen button as well as the hinged valve of the Provox voice prosthesis (Fig. 1). Biofilms causing the strongest increases in air flow resistance in case of Groningen button voice prostheses (see Table 1, Chapter 4 and Table 1, Chapter 5), will probably also cause the strongest increase in air flow resistance in case of Provox voice prostheses.

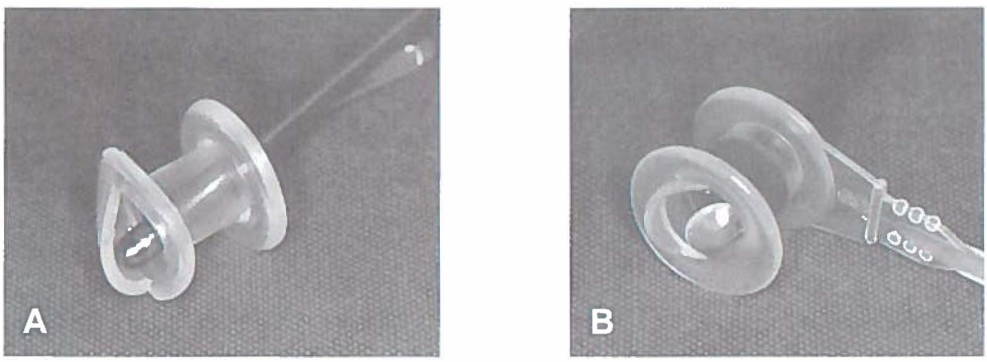

Figure 1. *A) The "Low Resistance" Groningen button voice prosthesis with opened slit valve. B) The Provox voice prosthesis with opened hinged valve.*

Method to study biofilm formation on voice prostheses

A suitable method to study biofilm formation on voice prostheses was developed by Leunisse *et al.*[1] and was used throughout this project. The only difference was the incorporation of a heating plate in the modified Robbins devices or artificial throats enabling to reach a temperature of 36°C to 37°C, comparable with the situation in a laryngectomized patient, whereas Leunisse *et al.*[1] carried out their experiments at room temperature. This is a significant modification of the experimental set-up, as the optimal growth conditions of oropharyngeal bacteria and yeasts are attained at temperatures of 34°C to 37°C and also the physico-chemical cell surface properties of bacteria and yeasts are dissimilar as compared with their growth at room temperature.[2] However, in the clinical situation voice prostheses are exposed to far more patient-related influences, such as saliva, food and drinks, acid reflux, medication, air flow and pressure mechanics of the esophageal wall. All these factors will have their impact on biofilm formation and can indeed be included in the model used. An important step to improve the experimental set-up would be to integrate the second dimension of valve failure, viz. "leakage through the voice prosthesis". Although the increase in air flow resistance caused by biofilm formation is easier and more reliable to measure as a dimension for failure of the valve mechanism, valve leakage is a more common clinical problem of malfunctioning prostheses and therefore of importance to assess.

A comparable experimental set-up can be used with the difference that in between flushing of the artificial throats the esophageal side of the prostheses will be connected with a column of water with a continuous pressure, to quantify leakage by measuring for example every 24 h the volume of water leaking through the voice prosthesis. At the end of the experiment both the increase in air flow resistance and the leakage through the voice prosthesis, caused by biofilm formation can be calculated.

Antimicrobial salivary peptides

Comparison of the results in Chapters 6 and 7 shows that the antimicrobial activity of the synthetic salivary peptides against biofilms formed in the modified Robbins devices is disappointing. Also, increases in concentration of the salivary peptide dhvar5 in Xialine did not result in less integrity of the biofilm and therefore diminished increases in air flow resistance. The chosen experimental set-up could be a problem, since prior to initiate

prevention of voice prosthetic failure because of biofilm formation by using antimicrobial peptides, antimicrobial mouthrinses or mucolytics, a mature biofilm was allowed to grow on the voice prostheses during three days, while under *in vivo* conditions drugs can be administered from the onset of biofilm formation. In fact, the study of Chapter 7 could be repeated by administering the "antibiofilm" therapy from the onset of biofilm formation. However, the reason to start applying antimicrobial peptides, antimicrobial mouthrinses or mucolytics *in vitro* on the moment a mature biofilm has been formed, is essential in our philosophy of patient treatment because the intention is not to chronically "medicalize" laryngectomized patients with a voice prosthesis but to use medication only a couple of days per month, or when voice prostheses related problems occur. In those situations a biofilm will be present and therefore it is of importance to determine the influence of "antibiofilm" therapy on a mature biofilm.

The limited effects of these synthetic salivary peptides on the integrity of the biofilm are likely due to the known antibiotic resistance of biofilms.[3] On the other hand, this explanation does not hold for the other applied antimicrobial agents evaluated, presumably because besides diffusion problems of "antibiofilm" agents from the surrounding medium, one agent will adsorb more readily to the biofilm during penetration than another.[3]

Future research

Prior to continue more extensive research *in vitro*, it would be of relevance to optimize the experimental set-up as described above. With the knowledge described in this thesis, the next step in the study on voice prosthetic biofilms should be to measure the two features of valve failure caused by biofilm formation, the increase in air flow resistance and leakage through the prosthesis. Subsequently, it would be interesting to mimic exactly the oral environment of a laryngectomized patient by, during the day, imitating the habits of eating and drinking, the salivary flow and composition, and time of speech. An optimized experimental set-up is essential to examine the methods of prolonging lifetimes of voice prostheses.

Studying the possibility of coating the esophageal side of voice prostheses, as done by Everaert *et al.*,[4] by using synthetic salivary peptides, could be an alternative approach to prolong the lifetime of voice prostheses. In case of synthetic salivary peptides, the problem is that the design of the peptide will only allow binding to the active domain of the peptide. Unfortunately, directly synthesizing salivary peptides to the esophageal side during

manufacturing of the peptides will not be possible because of the aggressive chemical agents used during synthesizing of the salivary peptides. Apparently, these antimicrobial peptides are less appropriate for application on mature biofilms consisting of bacterial and yeast strains. For that reason it would have been interesting to study *in vitro* the antimicrobial activity of dhvar4 and dhvar5, both added to Xialine, from the onset of biofilm formation, as until now no microbial resistance has been reported and therefore appropriate for frequent and long-term use. Although it is not the intention to chronically medicalize laryngectomized patients, also because daily use of chemicals to prevent biofilm formation will lead to high costs, dhvar4/Xialine and dhvar5/Xialine could be employed as a mouthspray for daily use from the moment a voice prosthesis is replaced.

Nowadays, an important topic in health care is "costs" and most of the decisions made by the secretary of public health and physicians are cost-related. This is probably the reason why in The Netherlands N-acetylcysteine has never been used by otolaryngologists to prevent voice prosthetic biofilm formation and malfunctioning, as laryngectomized patients have to bear the costs themselves. In a hypothetical case of a laryngectomized patient, with a mean voice prosthetic lifetime of 3 months, and assuming that by using acetylcysteine three times a day for 1 week per month, the prosthesis lifetime will be extended by 1 month, it can be calculated that this patient needs one voice prosthetic replacement procedure less per year, this indicates a saving of about 160 Euro. The use of N-acetylcysteine 3 weeks, three times a day (600 mg per day), will cost about 16 Euro. The gain, 144 Euro per year, multiplied by approximately 1800 laryngectomized patients relying on voice prostheses in The Netherlands, makes a clinical study into the effects of N-acetylcysteine worthwhile.

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Summary

Tracheoesophageal shunt speech is the most successful method in rehabilitating speech in laryngectomized patients. The majority of these patients are capable of using this method already within a number of days after laryngectomy and the quality of speech is high compared to the esophageal speech and the use of an electrolarynx, as alternative methods of speech rehabilitation. However, the limited lifetime of silicone rubber voice prostheses is worrisome and has to be conquered since each replacement bears the risk of inducing damage to the tracheoesophageal fistula. Moreover, replacements are regarded as very unpleasant by patients and also lead to high costs. The formation of a biofilm, consisting of bacteria and yeasts, on the esophageal side of voice prostheses causes malfunctioning of the valve mechanism and is generally held responsible for this limited lifetime. Therefore, particularly in case of laryngectomized patients with voice prosthetic lifetimes less than 3 to 4 months, there is need for employing "antibiofilm" therapy from the time of insertion of the voice prosthesis, preferably without using antimycotics or antibiotics because of the risk of inducing resistant strains.

Candida species are generally held responsible for failure of voice prostheses. Therefore, oropharyngeal yeast decontamination by using antifungal agents is a strategy frequently applied by various otolaryngologists, despite the fact that there is no compelling evidence to suggest that prescription of antifungal agents will actually prolong the lifetime of voice prostheses as concluded from a literature review, presented in **Chapter 1**. Moreover, the prophylactic use of antifungal agents may contribute to the development of resistant strains. Alternative approaches are proposed to prolong the lifetime of silicone rubber voice prostheses that may be found in modification of the silicone rubber surface of the implant, consumption of caffeinated soft drinks, diet supplementation with active, probiotic bacteria or salivary substitutes with synthetic antimicrobial peptides.

In **Chapter 2** a possible difference in biofilm composition of patients requiring frequent versus infrequent prosthesis replacements was investigated. Only Groningen button voice prostheses which were removed because of increased air flow resistance or leakage of food or liquids through the prosthesis were considered for this study. These prostheses were selected from a total of 692 failed voice prostheses over a 2 years evaluation period. The failed voice prostheses were subdivided into a short lifetime group, corresponding with an implantation-period less than 4 months (20 voice prostheses were included) and an extended lifetime group, comprising an implantation-period over 9 months (18 voice prostheses were included). The biofilm was removed from the valve sides of the prostheses. The bacterial

strain *Rothia dentocariosa* and the yeast strains *Candida albicans* and *Candida tropicalis* turned out to be predominant strains isolated from biofilms on voice prostheses in the short lifetime group, while in the extended lifetime group *R. dentocariosa* was found with a fourfold lower isolation frequency and *C. albicans* was found with a twofold lower isolation frequency. *C. tropicalis* was absent in the extended lifetime group.

Depending on tumor stage, treatment of laryngeal cancer consists of radiotherapy, surgery or both. In **Chapter 3** a possible relationship was investigated between voice prosthetic lifetime in laryngectomized patients, as limited by the occurrence of specific biofilms, and the irradiation dose applied to the neck node levels (field of the neck), in which the major salivary glands are partially included. Irradiation of the salivary glands can result in hyposalivation or a change of salivary properties, such as a reduced pH, buffer capacity, and the presence of specific non-immune and immune defense factors. Furthermore, a possible relationship between voice prosthetic lifetime and the irradiation dose applied to the primary tumor site was studied. To this end, a retrospective analysis was performed on 101 patients after total laryngectomy. The records of 101 patients who underwent a total laryngectomy between January 1993 and November 1999 at the Department of Otorhinolaryngology of the University Hospital Groningen, The Netherlands, were analysed. The following parameters were obtained: age, sex, radiotherapy, radiation fields, irradiation dose per field, tumor site, TNM classification and valve insertion. Irradiation to extensive neck fields, including the submandibular glands, did not influence the voice prosthetic lifetime after laryngectomy. However, primary tumor doses exceeding 60 Gray significantly shortened the mean voice prosthetic lifetime per patient. Hence, it can be hypothesized that specific biofilms limiting lifetime of voice prostheses develop as a result of radiation damage to the pharyngoesophageal segment.

Which bacterial or yeast strains, isolated from explanted voice prostheses, contribute most to increases in air flow resistance of silicone rubber voice prostheses, was determined in **Chapter 4**. Biofilms consisting either of a bacterial or a yeast strain, were grown on voice prostheses in the artificial throat model. The effects of these biofilms on air flow resistances were determined by calculating the difference in air flow resistance of individual voice prostheses as covered with a 7 days old biofilm with the situation prior to biofilm formation. Conspicuously, voice prosthetic biofilms formed by the bacterial strains *S. aureus* GB 2/1 and *R. dentocariosa* GBJ 41/25B and their excreted organic matter showed larger increases in air flow resistance (more then 30 cm H_2O.s/l) than biofilms formed by *Candida* species. This is

contrary to literature, where there seems to be agreement that *Candida* species are mainly responsible for clinical failure of silicone rubber voice prostheses.

The presence of particular combinations of bacterial and yeast strains in voice prosthetic biofilms has been suggested to be crucial for causing valve failure. In order to identify combinations of bacterial and yeast strains causative to failure of voice prostheses the effects of various combinations of bacterial and yeast strains on air flow resistances of Groningen button voice prostheses were determined in **Chapter 5**. Therefore, biofilms were grown on Groningen button voice prostheses by inoculating artificial throats with various combinations of clinically relevant bacterial and yeast strains. After 3 days, all throats were perfused three times daily with 250 ml phosphate buffered saline and at the end of each day the artificial throats were filled with growth medium during half an hour. After 7 days, the air flow resistances of the prostheses were measured. These air flow resistances were expressed relative to the air flow resistances of the same prosthesis prior to biofilm formation. This study shows that biofilms causing strong increases in air flow resistance (26 to 28 cm H_2O.s/l) were comprised of combinations, involving *C. tropicalis*, *S. aureus* and *R. dentocariosa*.

In **Chapter 6** the inhibitory effect of synthetic salivary antimicrobial peptides on the growth of bacteria and yeasts isolated from used silicone rubber voice prostheses was determined. To this end, the antimicrobial activities of 6 synthetic salivary peptides, (histatin 5, dhvar1, dhvar4, dhvar5, lactoferrin b 17-30 and cystatin S1-15) at concentrations of 2 mg/ml and 4 mg/ml were determined against different oropharyngeal yeast (n=4) and bacterial (n=8) strains and against a total microflora isolated from explanted voice prostheses using agar diffusion tests. The spectrum of susceptible microorganisms was determined qualitatively. It was found that histatin 5 and cystatin S1-15 did not show any antimicrobial activity against the microorganisms involved in this study. Dhvar1 was active against some of the oropharyngeal microorganisms tested, including the yeast strains but not against *R. dentocariosa*, *S. aureus*, *Escherichia coli* and the total microflora. Dhvar4 was active against all microorganisms tested, including the total microflora. Dhvar5 lacked activity against *E. coli* and the total microflora. Lactoferrin b 17-30 (LFb 17-30) did not inhibit the growth of any of the yeast strains involved and only showed minor activity against some of the bacterial strains. LFb 17-30 slightly inhibited the growth of the total microflora from an explanted prosthesis. This study shows that the synthetic salivary peptide dhvar4 has a broad antimicrobial activity against all microorganisms, commonly isolated from explanted voice prostheses, including yeasts. Therewith, it may represent a useful drug, as an alternative for

antibiotics and antimycotics employed in various ways to prolong the lifetime of voice prostheses in laryngectomized patients.

The integrity of biofilms on voice prostheses is critical to increases in air flow resistance of the valve mechanism of these prostheses. The integrity of a biofilm is determined in part by extracellular polymeric substances (EPS). As a result, increasing efforts have to be made by the patient in order to produce speech and prostheses have to be replaced regularly. The aim of the study described in **Chapter 7**, is to determine whether synthetic salivary peptides added to a salivary substitute or mucolytics influence the integrity of biofilms, consisting of bacteria and yeasts, as affecting the air flow resistance of voice prostheses. Phosphate buffered saline was chosen as a negative control, while the antiseptics chlorhexidine and triclosan were employed as positive controls. Biofilms were grown on Groningen button voice prostheses by inoculating an artificial throat model with a clinically relevant combination of bacterial and yeast strains during 3 days and subsequently exposing the biofilms three times per day to one of the chemicals evaluated. After 8 days, the percentages of viable bacteria and yeasts as well as the air flow resistances of the prostheses were measured relative to exposure to phosphate buffered saline. Synthetic salivary peptides added to a salivary substitute, nor salivary substitutes alone reduced the air flow resistance of voice prostheses after biofilm formation, despite the fact that one of the synthetic salivary peptides yielded a significant reduction in the percentage of viable organisms. Both chlorhexidine and triclosan reduced microbial numbers on the voice prostheses, but only the triclosan containing positive control reduced the air flow resistance of the prostheses. The mucolytic N-acetylcysteine induced a minor reduction in viable organisms on the prostheses, but yielded a significant decrease in air flow resistance showing the importance of EPS in maintaining the integrity of biofilms on voice prostheses, as affecting their air flow resistances.

In **Chapter 8**, the general discussion of this thesis, the reason for using the Groningen button voice prosthesis instead of the Provox voice prosthesis throughout the experiments *in vitro* performed in this thesis, is discussed. Furthermore, a possible method to improve the experimental set-up i.e. measuring both features of failure of the valve mechanism, including the increase in air flow resistance and leakage through the prosthesis, is described. Also possible reasons for the negative effects of synthetic salivary peptides on the integrity of the biofilm are proposed. Finally, suggestions are made for future research.

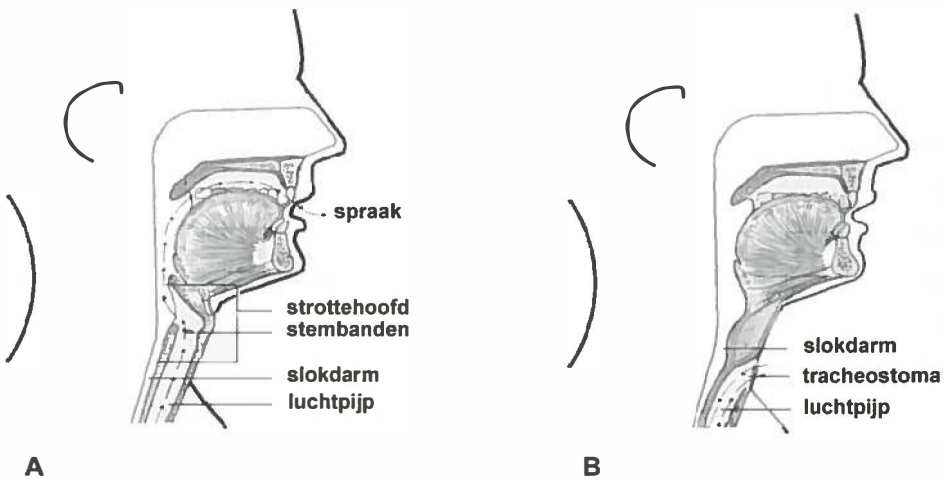
Samenvatting

Dysfunctie van het klepmechanisme van spraakprothesen als gevolg van biofilmvorming

Deze samenvatting is bedoeld om niet ingewijden in begrijpelijke taal uitleg te geven over het onderwerp van dit proefschrift en het verrichtte onderzoek. Gedetailleerde informatie is terug te vinden in de Engelstalige samenvatting van dit proefschrift.

Laryngectomie

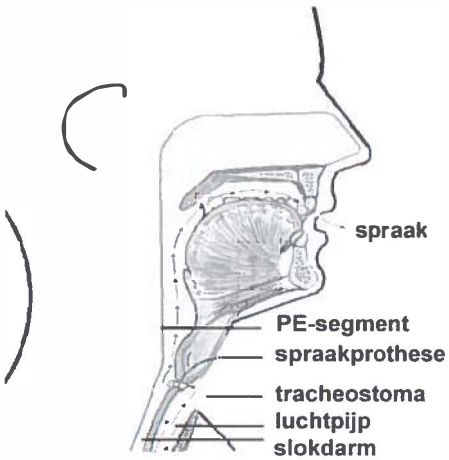
Een laryngectomie is een operatie die wordt uitgevoerd bij patiënten met kwaadaardige aandoeningen van het strottehoofd (larynx), die niet meer in aanmerking komen voor bestraling of waarbij de bestraling of microchirurgische behandeling niet voldoende effectief is gebleken en de aandoening is teruggekeerd. Bij deze operatie wordt het strottehoofd, inclusief stembanden (glottis), verwijderd en wordt de verbinding tussen de mond en de luchtpijp (trachea) opgeheven (zie Fig. 1). De ademhaling vindt nu plaats via een opening in de hals (tracheostoma). De verbinding tussen de mond en de slokdarm (oesophagus) is intact gehouden, waardoor de inname van voedsel en dranken via de normale weg kan blijven plaatsvinden. Het verlies van het vermogen tot spreken na deze operatie is sterk invaliderend en psychisch belastend. Laryngectomiepatiënten zijn dan ook gebaat bij een vlotte spraakrevalidatie.



Figuur 1. *A) Anatomie voor de laryngectomie. B) Anatomie na de laryngectomie.*

Spraakrevalidatie

Van de bestaande methoden voor het herstellen van spraak na laryngectomie, is de plaatsing van een spraakprothese in een, via chirurgie verkregen, verbinding tussen luchtpijp en slokdarm (tracheo-oesophageale fistel) het meest succesvol (zie Fig. 2). Door het tracheostoma met een duim of vinger af te sluiten, wordt lucht vanuit de longen gedwongen door de tracheo-oesophageale fistel te stromen richting de slokdarm. Deze luchtstroom zal het weefsel ter hoogte van de slokdarmingang (pseudoglottis of pharyngo-oesophageale (PE) segment) in trilling brengen en op deze manier kan geluid geproduceerd worden.

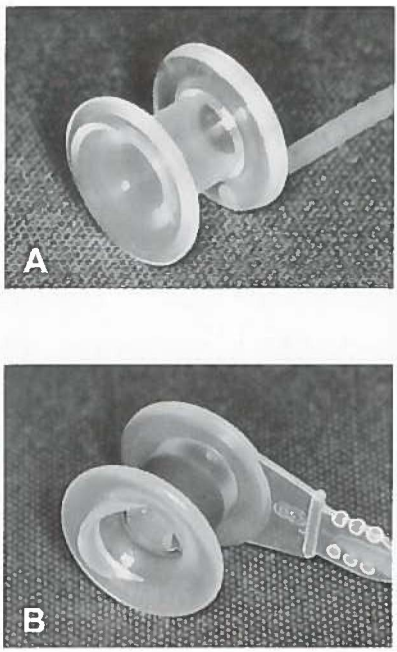


Figuur 2. *Anatomie na laryngectomie. Een spraakprothese is geplaatst in de verbinding die is gemaakt tussen de luchtpijp en de slokdarm.*

Spraakprothese

Een spraakprothese, gemaakt van siliconenrubber, heeft een klepmechanisme, dat passage van uitgeademde lucht vanuit de luchtpijp naar de slokdarm mogelijk maakt, maar dat tegelijkertijd voorkomt dat er speeksel, drank of voedsel vanuit de slokdarm in de luchtpijp lekt. Tevens dient deze spraakprothese ter preventie van spontane sluiting van de verbinding zoals gemaakt tussen de luchtpijp en slokdarm (tracheo-oesophageale fistel).

Er zijn in de loop der jaren diverse typen spraakprothesen op de markt gebracht. De op dit moment in Nederland meest frequent gebruikte spraakprothesen zijn de Groningen button (Fig. 3A) en de Provox spraakprothese (Fig. 3B).

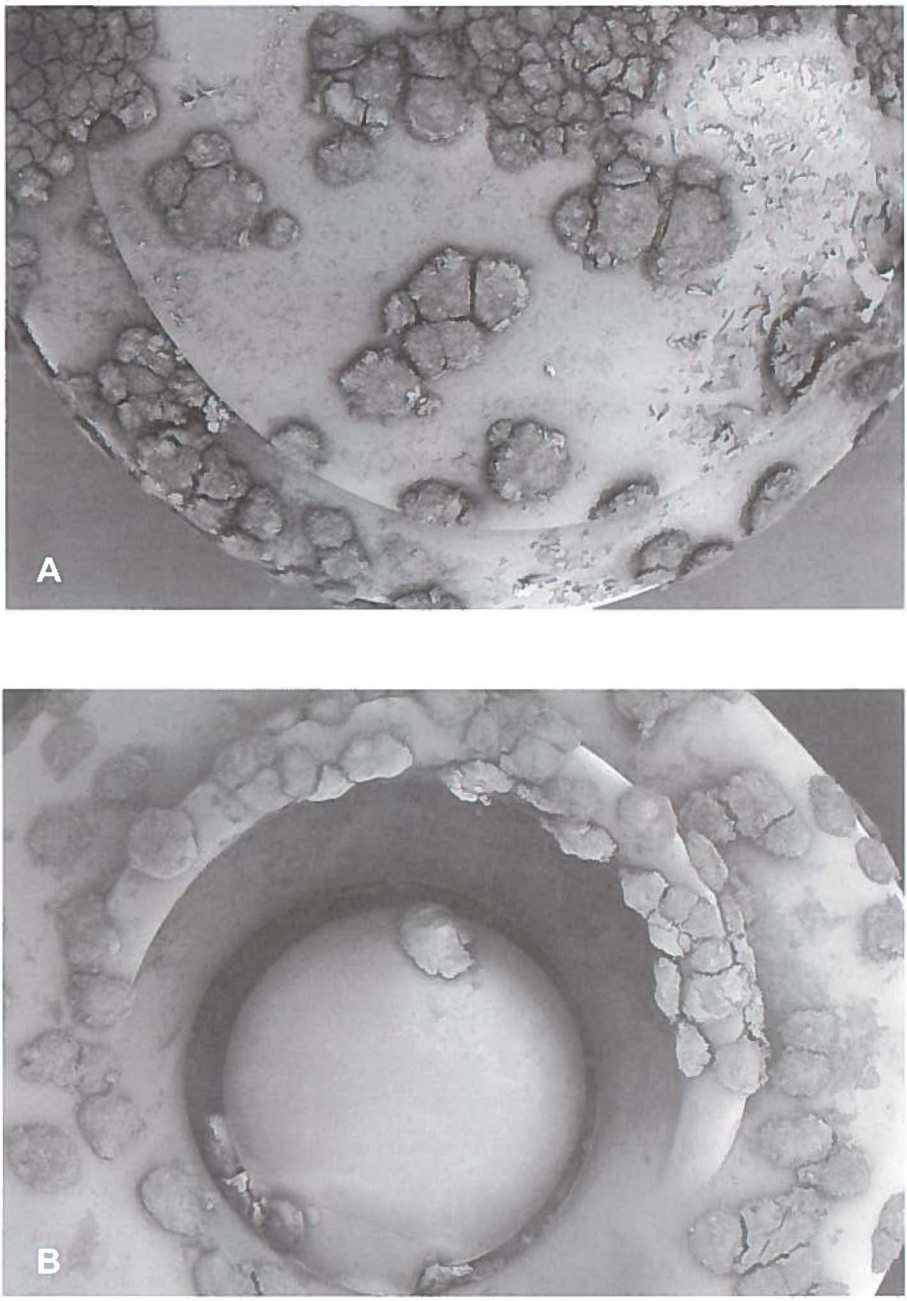


Figuur 3. *A) Groningen button spraakprothese. B) Provox spraakprothese.*

Spraakprothese en levensduur

De levensduur van siliconenrubber spraakprothesen is beperkt, doordat er zich bacteriën en gisten gaan hechten (biofilmvorming) op het oppervlak van de prothese, met name aan de slokdarmzijde (oesophageale zijde) (zie Fig. 4). Hierdoor wordt het klepmechanisme van de spraakprothese belemmerd in zijn functioneren en zal lekkage optreden van speeksel, voedsel of drank vanuit de slokdarm in de luchtpijp of zal de luchtstroomweerstand toenemen waardoor het spreken steeds moeizamer verloopt. In beide gevallen zal de spraakprothese poliklinisch verwisseld moeten worden door een keel-, neus- en oorarts.

De gemiddelde levensduur van siliconenrubber spraakprothesen is 3 tot 4 maanden, maar de levensduur van spraakprothesen kan van patiënt tot patiënt sterk verschillen. Een wisseling van de prothese wordt als 'erg vervelend' ervaren door de laryngectomiepatiënten en bovendien betekent elke wisseling een reis naar de polikliniek. Ook is iedere prothese-wisseling een extra belasting voor de tracheo-oesophageale fistel, die na een aantal wisselingen niet meer goed zal sluiten rondom de prothese en er vervolgens lekkage kan gaan optreden rondom de prothese. Het vinden van een methode die de levensduur van spraakprothesen kan verlengen, zal een belangrijke positieve invloed hebben op de kwaliteit van leven van laryngectomiepatiënten.



Figuur 4. *Biofilmvorming op de slokdarmzijde van de spraakprothese. A) Groningen button spraakprothese geëxplanteerd na 4 maanden. B) Provox spraakprothese geëxplanteerd na 6 maanden.*

Wat is een biofilm?

Bacteriën en/of gisten die zich hechten aan het oppervlak van een biomateriaal zoals een spraakprothese, urinewegcatheter, contactlens of kunstheup, weten te overleven doordat zij een biofilm vormen. Een biofilm is opgebouwd uit een matrix van bacteriën en/of gisten, die aan de ene zijde beschermd wordt door het oppervlak van het biomateriaal en aan de andere zijde door een slijmachtige laag geproduceerd door de bacteriën en/of gisten. Een biofilm beschermt zich op deze manier tegen invloeden van buitenaf. Antigist medicatie (antimycotica) of antibiotica zullen weinig effect hebben aangezien de laag van de biofilm die zich heeft gehecht aan de zijde van het biomateriaal niet bereikt kan worden. Bacteriën en/of gisten die zich in deze laag van de biofilm bevinden, kunnen zich dus ongehinderd blijven vermenigvuldigen. Een geïnfecteerd biomateriaal maakt veelal verwijdering van dit biomateriaal noodzakelijk.

Korte versus lange levensduur van spraakprothesen

Gezien de grote verschillen in levensduur van spraakprothesen, is in eerste instantie onderzocht of dit verschil in levensduur verband houdt met een verschil in microbiële samenstelling van de biofilm die zich aan het oppervlak hecht. Hiervoor zijn dysfunctionele spraakprothesen van patiënten verzameld, die korter dan 4 maanden of langer dan 9 maanden functioneel zijn geweest. De verschillende bacteriën en gisten, waaruit de aangetroffen biofilms waren opgebouwd, zijn geïdentificeerd. Hieruit bleek dat in biofilms gevormd op spraakprothesen met een levensduur korter dan 4 maanden, de gisten *Candida albicans* en *Candida tropicalis* en de bacterie *Rothia dentocariosa* frequenter te vinden zijn.

Bestraling van speekselklieren

De meeste laryngectomiepatiënten zijn bestraald in het hoofd-hals gebied. De bestralingsvelden kunnen worden opgedeeld in twee delen, het grote en het kleine veld. Bij grotere tumoren wordt in de meeste gevallen het grote veld bestraald, dat wil zeggen het primaire tumorgebied en de lymfeklieren in de hals. Binnen dit grote veld liggen tevens de gehele onderkaakspeekselklier (glandula submandibularis) en een deel van de oorspeekselklier (glandula parotis), waarbij dysfunctie kan optreden door de bestraling. Bij dysfunctie is de speekselvloed afgenomen, waardoor er onder andere een verstoring in de orale microflora en in de antimicrobiële speekseleiwitten kan ontstaan. Deze speekseleiwitten beschermen de weefsels in de mond-keelholte tegen infecties door bacteriën, gisten en

virussen. Slechts een kleine groep van de laryngectomiepatiënten is enkel en alleen op het primaire tumorgebied, het kleine veld, bestraald.

De medische gegevens van 101 patiënten die een laryngectomie hebben ondergaan en tevens een spraakprothese kregen, werden onderzocht. Er is gekeken naar een mogelijke relatie tussen eventuele dysfunctie van de grote speekselklieren door bestraling en een beperkte levensduur van spraakprothesen. Echter deze relatie kon niet worden aangetoond. Daarentegen werd er wel een verband aangetoond tussen bestraling op het primaire tumorgebied, zoals het geval bij het grote en kleine veld, en levensduur van prothesen. Indien de bestralingsdosis op het primaire tumorgebied groter of gelijk aan 60 Gray was, bleek de levensduur van spraakprothesen significant korter te zijn.

Mogelijkerwijs veroorzaakt een bestralingsdosis op het primaire tumorgebied die groter of gelijk is aan 60 Gray, dysfunctie van de kleine speekselkliertjes van het pharyngo-oesophageale segment, waardoor er een lokale verstoring in de microflora ontstaat en de levensduur van spraakprothesen in negatieve zin beïnvloed wordt.

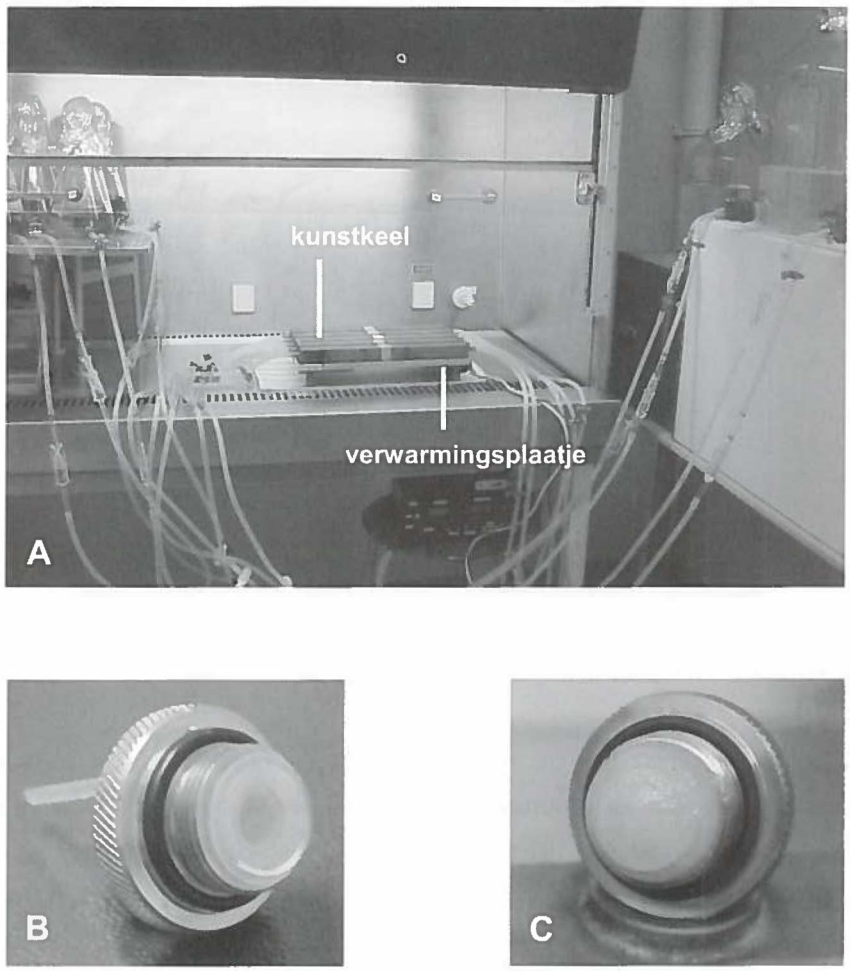
De gist Candida en antigist medicatie

Dagelijks komt de mond-keelholte in aanraking met veel soorten micro-organismen (bacteriën en gisten), die dankzij in het speeksel aanwezige antimicrobiële afweermechanismen voor het overgrote deel belemmerd worden in hun kolonisatie op weefsels in de mond-keelholte (tanden, slijmvliezen). Uit biofilmonderzoek betreffende dysfunctionele spraakprothesen van patiënten is gebleken dat een biofilm altijd is opgebouwd uit een combinatie van bacteriën en gisten. Echter, de gist *Candida* wordt door veel onderzoekers gezien als oorzaak van aantasting van siliconenrubber spraakprothesen, waardoor dysfunctie van het klepmechanisme optreedt. Deze gedachte is gebaseerd op (elektronen)microscopische opnames en kweken van biofilms, die met name *Candida* aantoonden in de biofilm.

In de kliniek worden bij laryngectomiepatiënten met een zeer korte levensduur van hun spraakprothese *Candida* infecties bestreden met orale antimycotica. Gebleken is echter dat het toepassen van antimycotica niet tot een significante verlenging van de levensduur van siliconenrubber spraakprothesen leidt. Het levert slechts een tijdelijke verbetering op van het orale milieu.

Kunstkeel

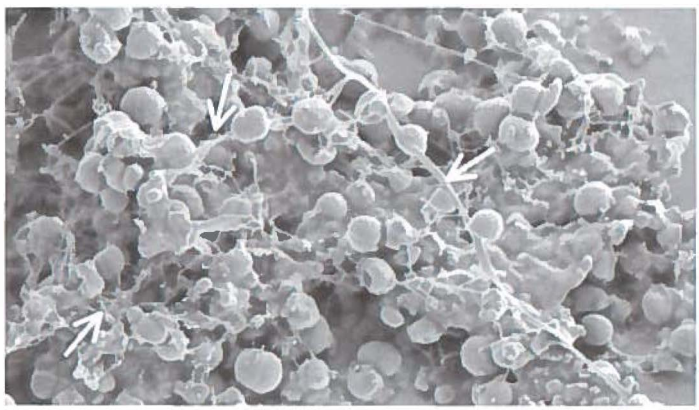
Om in het laboratorium het proces van biofilmvorming op spraakprothesen te kunnen nabootsen, werd gebruik gemaakt van de kunstkeel (zie Fig. 5). De kunstkeel is een roestvrijstalen buis, die op lichaamstemperatuur wordt gehouden en waarin spraakprothesen geplaatst kunnen worden. Met behulp van deze opstelling is het mogelijk binnen een aantal dagen een biofilm te laten groeien, die niet te onderscheiden is van een biofilm op een spraakprothese van een laryngectomiepatiënt.



Figuur 5. *A) Opstelling van de kunstkeel. B) Bougie met een ongebruikte spraakprothese, die in de kunstkeel geplaatst kan worden. C) Bougie met een spraakprothese, waarop zich een biofilm heeft gevormd.*

Biofilmonderzoek in de kunstkeel

Om de invloed van diverse gist- en bacteriestammen op siliconenrubber spraakprothesen te onderzoeken, werden biofilms bestaande uit een enkele bacterie- of giststam op spraakprothesen in de kunstkeel gekweekt. De mate van dysfunctie van het klepmechanisme van de spraakprothese door biofilmvorming werd onderzocht aan de hand van electronenmicroscopische opnames van de prothese en de verandering in luchtstroomweerstand van de spraakprothese door biofilmvorming. Electronenmicroscopische opnames lieten zien dat bacteriën in een biofilm onderling verbonden zijn door slijmachtige structuren (zie Fig. 6). De biofilms gevormd door de bacterie *Staphylococcus aureus* of *Rothia dentocariosa* bleken in staat tot een grotere toename in luchtstroomweerstand dan een biofilm gevormd door enkel *Candida*.



***Figuur 6**. Bacteriën in een biofilm onderling verbonden door slijmachtige structuren.*

Vervolgens werden biofilms bestaande uit diverse combinaties van bacteriën en gisten, zoals bij laryngectomiepatiënten het geval is, op de spraakprothesen in de kunstkeel gekweekt. Uit dit onderzoek is gebleken dat specifieke combinaties van bacteriën en gisten in de biofilm wel degelijk invloed hebben op de levensduur van spraakprothesen. Echter, het aantal bacteriën en gisten in een biofilm, dus de dikte van de biofilmlaag op de prothese, lijkt geen rol te spelen.

Antimicrobiële speekseleiwitten

In het speeksel van de mens zijn verschillende soorten afweersystemen tegen bacteriën en gisten aanwezig. Onder andere de antimicrobiële speekseleiwitten behoren tot deze afweersystemen en zijn al duizenden jaren werkzaam zonder dat er resistentie bij de mens is opgetreden. Veel laryngectomiepatiënten zijn bestraald in het hoofd-hals gebied en als bijwerking van deze bestraling kan de speekselvloed verminderd zijn en daarmee ook de antimicrobiële activiteit van het speeksel. Deze patiënten zijn dan ontvankelijker voor infecties in de mond-keelholte, die optreden als gevolg van veranderingen in de samenstelling van bacteriën en gisten in diezelfde mond-keelholte. Antimicrobiële speekseleiwitten zoals histatine, cystatine en lactoferrine hebben zowel een antibacteriële als antigist werking en zouden bij laryngectomiepatiënten gebruikt kunnen worden als toevoeging aan bijvoorbeeld kunstspeeksel.

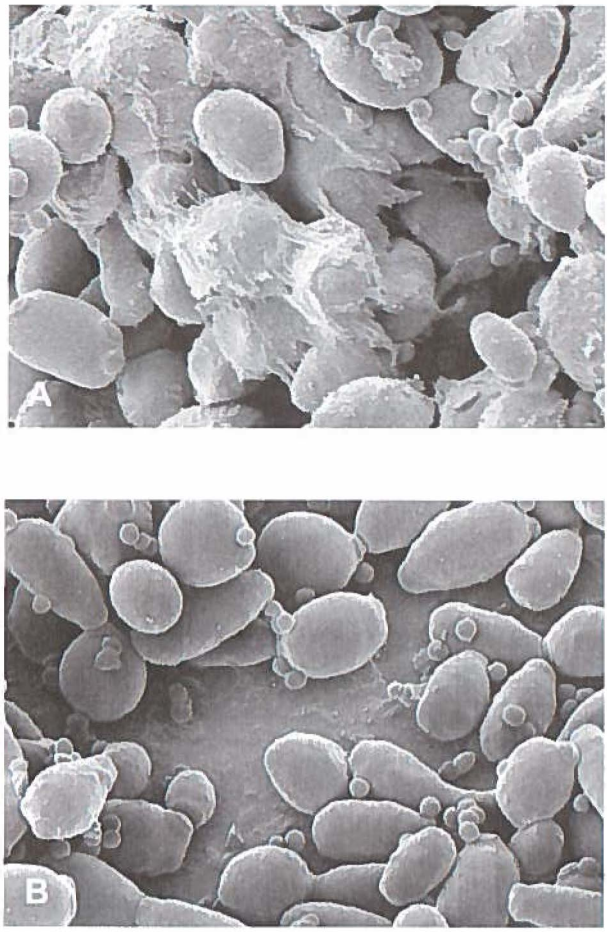
In het laboratorium is onderzoek gedaan naar het mogelijk remmend effect van speekseleiwitten op de groei van bacteriën en gisten zoals die werden geïsoleerd van gebruikte dysfunctionele spraakprothesen. De synthetische speekseleiwitten dhvar4 en dhvar5, varianten op het natuurlijke histatine, blijken een duidelijk remmend effect te vertonen op micro-organismen geïsoleerd uit biofilms van spraakprothesen.

Vervolgens is met behulp van de kunstkeel gekeken naar de mate van dysfunctie van het klepmechanisme van de spraakprothese door biofilmvorming, beïnvloed door speekseleiwitten toegevoegd aan kunstspeeksel. Dit is onderzocht aan de hand van electronenmicroscopische opnames van de prothese en de verandering in luchtstroomweerstand van de spraakprothese door biofilmvorming. De resultaten van dit experiment in de kunstkeel geven geen aanwijzing dat de toevoeging van antimicrobiële speekseleiwitten aan kunstspeeksel een bijdrage levert aan de verlenging van de levensduur van spraakprothesen.

Slijmoplossend middel

Uit het verrichtte onderzoek en na bestuderen van electronenmicroscopische opnames van biofilms op dysfunctionele spraakprothesen is gebleken dat slijmproducerende bacteriën een mogelijk belangrijke rol spelen in de sterke samenhang van biofilms, waardoor dysfunctie van het klepmechanisme ontstaat. Tevens biedt deze slijmlaag bescherming tegen invloeden van buitenaf, zoals antibacteriële of antigist medicatie. Met behulp van de kunstkeel is onderzoek gedaan naar de mate van dysfunctie van het klepmechanisme van de spraakprothese door

biofilmvorming, beïnvloed door een slijmoplossend middel (N-acetylcysteïne). Electronenmicroscopische foto's zijn gemaakt en bovendien is gekeken naar de verandering in luchtstroomweerstand van de prothese door biofilmvorming beïnvloed door een slijmoplossend middel. Deze slijmoplossende middelen lijken een zodanige verstoring te veroorzaken in de samenhang van de biofilm (vergelijk Fig. 7A en Fig. 7B), dat een positief effect kan worden verwacht op de levensduur van spraakprothesen.



Figuur 7. *Biofilmvorming op een spraakprothese uit de kunstkeel. A) De controle. B) Een qua microbiële samenstelling identieke biofilm, echter deze biofilm is blootgesteld geweest aan een slijmoplossend middel.*

Samengevat kan worden gesteld dat een bestralingsdosis van 60 Gray of meer op het primaire tumorgebied een zodanige verstoring geeft in de microflora van het pharyngo-oesophageale segment, dat dit milieu met name geschikt is voor slijmproducerende bacteriën die voor een sterke samenhang binnen de biofilm zorgen en daardoor een negatief effect hebben op de levensduur van spraakprothesen. De in dit proefschrift verder uitgewerkte microbiologie van de kunstkeel stelt ons in staat onderzoek te doen naar middelen die biofilmvorming op spraakprothesen voorkomen. Van de middelen die in dit proefschrift onderzocht zijn, blijken met name slijmoplossende middelen (N-acetylcysteïne) een gunstig effect te hebben op de levensduur van spraakprothesen.

Publications Related to the Subject

1. Elving GJ, Van der Mei HC, Busscher HJ, Nieuw Amerongen AV, Veerman ECI, Van Weissenbruch R, Albers FWJ. Antimicrobial activity of synthetic salivary peptides against voice prosthetic microorganisms. *Laryngoscope* 2000;110:321-4.

2. Elving GJ, Van der Mei HC, Van Weissenbruch R, Albers FWJ, Busscher HJ. Effect of antifungal agents on indwelling voice prosthetic biofilms. *Current Opinion in Otolaryngology & Head and Neck Surgery* 2000;8:165-8.

3. Elving GJ, Van der Mei HC, Busscher HJ, Van Weissenbruch R, Albers FWJ. Air flow resistances of silicone rubber voice prostheses after formation of bacterial and fungal biofilms. *Journal of Biomedical Materials Research (Applied Biomaterials)* 2001;58:421-6.

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5. Elving GJ, Van der Mei HC, Busscher HJ, Van Weissenbruch R, Albers FWJ. A comparison of the microbial composition of voice prosthetic biofilms from patients requiring frequent versus infrequent replacements. *Annals of Otology, Rhinology & Laryngology* 2001, in press.

6. Elving GJ, Stokroos I, Nieuw Amerongen AV, Van der Mei HC, Busscher HJ, Van Weissenbruch R, Albers FWJ. The influence of antimicrobial peptides and mucolytics on the integrity of biofilms consisting of bacteria and yeasts as affecting voice prosthetic air flow resistances. *Journal of Medical Microbiology* 2001, submitted.

7. Elving GJ, Van Weissenbruch R, Burlage FR, Busscher HJ, Van der Mei HC, Albers FWJ. The influence of radiotherapy on the lifetime of silicone rubber voice prostheses in laryngectomized patients. *Laryngoscope* 2001, submitted.

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9. Van der Mei HC, Free RH, Elving GJ, Van Weissenbruch R, Albers FWJ, Busscher HJ. Effect of probiotic bacteria on prevalence of yeasts in oropharyngeal biofilms on silicone rubber voice prostheses *in vitro*. *Journal of Medical Microbiology* 2000;49:713-8.

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Curriculum Vitae

G. Jolanda Elving was born on January 2, 1971 in Coevorden, The Netherlands.

After obtaining her Atheneum-B diploma at the Rijksscholengemeenschap Coevorden in 1990, she studied physiotherapy for one year at the Rijkshogeschool Groningen, The Netherlands. In 1991 she started to study medicine at the University of Groningen, The Netherlands, where she graduated in 1998.

In september 1998 she started her thesis "Voice prosthetic valve failure due to biofilm formation" in cooperation with the Department of Otorhinolaryngology of the University Hospital Groningen, The Netherlands (Head: Prof. dr. F.W.J. Albers) and the Department of Biomedical Engineering of the University of Groningen, The Netherlands (Head: Prof. dr. ir. H.J. Busscher). Most of the work described in this thesis was performed at the Department of Biomedical Engineering of the University of Groningen. In september 2000 the NWO (Nederlandse Organisatie voor Wetenschappelijk Onderzoek) granted her the AGIKO (Assistent Geneeskundige In opleiding tot Klinisch Onderzoeker) scholarship. In may 2002 she will start her residency in Otorhinolaryngology in the Department of Otorhinolaryngology of the University Hospital Groningen.

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